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(54) Title: METHOD AND APPARATUS FOR DETERMINING TARGET DEPTH, BRIGHTNESS AND SIZE WITHIN A BODY REGION

(57) Abstract: A method of investigating the location and size of a light-emitting source in a subject is disclosed. In practicing the method, one first obtains a light intensity profile by measuring, from a first perspective with a photodetector device, photons which (i) originate from the light-emitting source, (ii) travel through turbid biological tissue of the subject, and (iii) are emitted from a first surface region of interest of the subject. The light-intensity profile is matched against with a parameter-based biophotonic function, to estimate function parameters such as depth and size. The parameters so determined are refined using data other than the first measured light intensity profile, to obtain an approximate depth and size of the source in the subject. Also disclosed is an apparatus for carrying out the method.



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METHOD AND APPARATUS FOR DETERMINING
TARGET DEPTH, BRIGHTNESS AND SIZE WITHIN A BODY REGION

Field of the invention

5 This invention relates to an apparatus and method for fields of non-invasive medical imaging, medical research, pathology, and drug discovery and development.

Background of the invention

10 In a variety of medical diagnostic and therapeutic settings, as well as in biomedical research, it is desirable to image a subsurface target or area within a body region of a subject. For example, non-invasive locating and imaging of part or all of a solid tumor, an area of myocardial ischemia, the distribution of a therapeutic compound administered to the subject, or the progression of a
15 disease may provide useful research or diagnostic information. Ideally, an imaging method is able to locate within a body region a target of interest, and provide information about the target's shape, size, number of cells, and depth below the surface of the body region. However, until now, methods that have been used and/or proposed for subsurface body target imaging have generally
20 been limited to those using ionizing radiation such as X-rays, expensive and bulky equipment such as Magnetic Resonance imaging (MRI), or ultra-sound.

 X-rays have excellent tissue penetration, and when used in conjunction with computed tomography (CT) or computed axial tomography (CAT), can produce superior image quality. However, X-rays have limited use in monitoring
25 disease progress because exposure to X-rays is potentially harmful if such exposure is prolonged. X-rays can be used to locate and image compositions that have localized at a target within a body region, but always with exposure to the potential harm associated with X-ray radiation. X-rays, however, cannot readily be used to image the expression of gene products *in vivo* and determine
30 the depth and/or shape of a target expressing such gene products.

 MRI is also an excellent method for imaging targets, areas, and structures in a body region of a subject. Although MRI is not thought to possess harmful

attributes like those associated with ionizing radiation, the expense and bulky equipment size needed to use MRI make it impractical for many applications or situations. MRI can provide two and three-dimensional information about targets within a body region of a subject, but is less effective at imaging physiological activity associated with a target.

Ultrasound or ultrasonography is the use of high-frequency sound (ultrasonic) waves to produce images of structures within the human body. Ultrasonic waves are sound waves that are above the range of sound audible to humans. Ultrasonic waves are produced by the electrical stimulation of a piezoelectric crystal and such waves can be aimed at specific body regions. As the waves travel through body tissues within a body region, they are reflected back at any point where there is a change in tissue density, as, for instance, in the border between two different organs of the body. Ultrasound offers the advantages of not using radiation or radioactive material, and employs lesser expensive and less bulky equipment than MRI, but is limited to only discerning differences in density of underlying tissue and structures. Accordingly, ultrasound cannot effectively track and monitor the progress of an infection unless such infection results in a discernable shift in density of the target tissue. Ultrasound cannot image or detect the physiological functions of tissues or organs.

Until now, Positron Emission Tomography or P.E.T. was unique among imaging techniques because it produces an image of organ or tissue function. Other imaging techniques such as X-ray, CT, MRI, and sonography depict organ or tissue anatomy but cannot discern physiological activity within them. To image a specific biochemical activity of an organ, a radioactive substance, called a radiotracer or radiopharmaceutical, is injected into the body or inhaled. The tracer is usually a radioactive equivalent of a substance that occurs naturally within the body such as water or sugar. The radioactive isotope is identical to the body's own nonradioactive isotope except that its atoms have a different number of neutrons. Consequently, a subject's body is burdened with radioactive material, and the potential harm associated with such material. P.E.T cannot detect non-isotopic expression products from transgenic tissues, organs, or

transgenic organisms. Scintigraphy, a diagnostic technique in which a two-dimensional picture of a bodily radiation source is obtained by the use of radioisotopes, may also be used for imaging structures and their functions. Scintigraphy, however, is not suitable for determining the depth of a target in a body region of a subject.

In view of the above-mentioned technologies for locating and imaging a target in a body region of a subject, there is a need for methods and devices to determine the depth and/or the shape and/or number of cells of such target without having to use radioactivity, radiation, or expensive and bulky equipment.

The invention disclosed herein meets these needs.

Summary of the Invention

In one aspect, the invention includes a method of investigating the location, size and number of cells, of a light-emitting source in a subject. In practicing the method, one initially obtains a first measured light intensity profile constructed by measuring, from a first perspective with a photodetector device, photons which (i) originate from the light-emitting source, (ii) travel through turbid biological tissue of the subject, and (iii) are emitted from a first surface region of interest of the subject. The light-intensity profile is matched against a parameter-based biophotonic function to estimate function parameters such as depth and size. The parameters so determined are refined using data other than the first measured light intensity profile, to obtain an approximate depth and size of the source in the subject. The additional data may be data measured from the subject, data from modeling analysis, or data relating to the wavelength of photons emitted from the surface of the subject. As examples:

The method typically includes generating a 2-D or 3-D visual representation of the light-emitting source, using the approximate depth and shape of the source in the subject, and superimposing the visual representation onto a 2-D or 3-D image of the subject.

The additional data may be obtained from computer simulation of the diffusion of light from a light-emitting source in a turbid medium. One preferred simulation approach involves (i) generating a plurality of theoretical light-intensity

profiles, based on a model of photon diffusion from a glowing source located at one of a plurality of depths, and having one of a plurality of sizes and shapes, through a turbid medium having absorption and scattering properties similar to those of biological tissue, (ii) comparing the quality of fit between each of the plurality of theoretical light-intensity profiles and the first measured light intensity profile, (iii) selecting the theoretical light intensity profile which provides to the first measured light intensity profile, and (iv) obtaining an approximate depth, shape and brightness of the source in the subject using parameters from the theoretical light intensity profile selected in (iii). The method may include employing in a photon-scattering model, one or more predetermined tissue-specific light-scattering coefficients corresponding to tissue through which the photons travel.

In another general embodiment, the additional data are obtained by measuring light emission from the subject at two or more different wavelengths between about 400 nm and about 1000 nm, determining the relative light intensities measured at the different wavelengths, and comparing the determined relative light intensities with known relative signal intensities at the different wavelengths, as a function of tissue depth. Alternatively, the spectrum of light intensities is measured over a selected wavelength range between about 400-1000 nm, and the measured spectrum is compared with a plurality of spectra measured from light-emitting sources placed at various depths within tissue, to determine the depth of the light-emitting source from matching the measured spectrum with the known spectra.

In various embodiments for a light-emission source, the source is a luminescent moiety or a fluorescent moiety; the light-emitting source is administered to the subject and binds to a selected target or is otherwise localized in the subject prior to the measuring; and the light-emitting source is a light-generating protein (e.g., a luciferase, a green fluorescent protein, etc.), such as a light-generating protein expressed by biological cells of the subject or biological cells administered to the subject.

In another aspect, the invention includes apparatus for use in investigating the location and size of a light-emitting source in a subject. The

apparatus includes a light-tight enclosure within which light-emission events from the subject can be detected and an optical system associated with the enclosure for use in generating a first light intensity profile constructed by measuring, from a first perspective within the enclosure, photons which (i) originate from the light-emitting source, (ii) travel through turbid biological tissue of the subject, and (iii) are emitted from a first surface region of interest of the subject. A computational unit operatively connected to the photo-detector functions to (i) fit the first measured light intensity profile with a parameter-based biophotonic function; and (ii) refine the parameters of the biophotonic function, using data other than the first measured light intensity profile, to generate an approximate depth and shape of the source in the subject.

The optical system preferably includes an intensified or cooled charge-coupled-device (CCD), and a lens for focusing light onto the CCD. The optical system may be configured in such a way as to detect photons emitted from a plurality of different selected surface regions of interest of the subject. The system may include one or more filters for transmitting photons within different selected wavelength ranges, *e.g.*, above and below 600 nm.

The computational unit may include a data file of model biophotonic functions representing light-emitting sources of various sizes at various depths, for curve matching with the first spatial profile.

Where the optical system includes filters for wavelength discrimination, the computation unit is operable to carry out at least one of the parameter-refinement operations:

(i) determining the relative light intensities measured at the different wavelengths, and comparing the determined relative light intensities with known or calculated relative signal intensities at the different wavelengths, as a function of tissue depth; and

(ii) comparing the measured spectrum with a plurality of spectra measured from light-emitting sources placed at various depths within tissue, and determining the depth of the light-emitting source from matching the measured spectrum with the known spectra.

In another embodiment, the computational unit is operable to average the intensity pattern image into a single intensity value, and further to determine source size by integrated light intensity values generated as a function of a light-emitting source of a particular size and shape.

5 The computational unit may have a database containing a plurality of theoretical light-intensity profiles, based on a model of photon diffusion from a light-emitting source located at one of a plurality of depths, and having one of a plurality of sizes and shapes, through a turbid medium having absorption and scattering properties similar to those of biological tissue. Here the computational
10 unit is operable to (i) compare the quality of fit between each of the plurality of theoretical light-intensity profiles and first measured light intensity profile, (ii) select the theoretical light intensity profile which provides the best fit to the first measured light intensity profile, and (iii) obtain an approximate depth and shape of the source in the subject using parameters from the theoretical light intensity
15 profile selected in (ii).

In addition, the computational unit is operable to generate a visual 2-, or 3-dimensional representation of the light-emitting source, using the approximate depth and shape of the source in the subject, and superimposing the visual representation onto a 2- or 3-dimensional image of the subject.

20 In another aspect, the invention provides a method of determining the depth of a light-emitting source in a subject. In practicing the method, the light emission intensity from the subject at two or more different wavelengths between about 400 and about 1000 nm is measured. The depth of the light-emitting source is determined using the measured light intensities and information related
25 to the optical properties of the subject's tissue, *e.g.*, coefficients of attenuation and diffusion in the subject.

Information relating to optical properties, *e.g.*, the coefficients of attenuation and diffusion in the subject, may be obtained by direct measurement of the coefficients in material that is the same or similar to that of the subject.

30 Alternatively, optical-properties information may be obtained indirectly, by determining, at two or more different wavelengths, light intensities from a light-emitting source located at each of a plurality of depths in tissue or material

corresponding to that of the subject. The desired depth determination is then carried out by matching the measured light intensities with the light intensities determined at each of the plurality of depths.

5 In the latter approach, the spectral profile of light intensities from the light-emitting source may be compared (matched) with each of a plurality of spectral profiles of light intensities from a light-emitting source at each of a plurality of depths.

10 These and other objects and features of the invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

Brief Description of the Drawings

15 Fig. 1A is a cross-sectional view of an apparatus constructed in accordance with the invention, for use in investigating the location and size of a light-emitting source in a subject;

Fig. 1B illustrates features of the apparatus in Fig. 1A in schematic form;

Fig. 2 is a flowchart of the general steps that can be carried out by the apparatus, in practicing the method of the invention;

20 Figs. 3A is a surface light-intensity image from a light-emitting source in an animal subject.

Fig. 3B is a light emission profile from the animal subject in Fig. 3A, showing the curve fit between the measured emission profile (solid lines) and a light-emission profile calculated from a diffusion model;

25 Figs. 4A and 4B are plots of the emission spectra of bacterial luciferase *in vitro* (solid line), and *in vivo* (dashed lines) at various depths in a mouse thigh, as indicated, where the plot in Fig. 4B has been scaled to show the peak intensity *in vivo* in the region between 600-700 nm;

Fig. 5 shows various locations in an animal at which transmission spectra were obtained.

30 Figs. 6A and 6B show transmission spectra through a live experimental animal at the various animal locations shown in Fig. 5;

Fig. 6C illustrates a double integrating sphere apparatus useful for measuring optical properties of a sample, e.g., tissue;

Fig. 6D illustrates the experimental set up of a radial fiber probe, useful for measuring optical properties of tissue *in vivo*;

5 Figs. 7A and 7B are plots of the absorption coefficients;

Figs. 7C and 7D are plots of the isotropic or reduced scattering coefficients of various tissues as a function of wavelength;

Fig. 8A illustrates components of the apparatus used for absolute calibration determination;

10 Fig. 8B illustrates how absolute calibration allows determination of cell count from an animal subject;

Figs. 9A and 9B illustrate how photons diffuse through tissue (9A) and how the photons that diffuse to the surface of the tissue are captured in the invention (9B);

15 Fig. 10A shows a Monte Carlo simulation following a photon through a "random walk" through turbid tissue;

Fig. 10B illustrates a calculated light intensity spatial profile calculated for a light source at 4 mm depth from the diffusion model (solid line) and with Monte Carlo simulations ("+" symbols);

20 Figs. 11A and 11B are plots calculated from the diffusion model showing peak intensity (11A) and spot width (FWHM) (10B) as a function of source depth;

Fig. 12A is a surface light-intensity image from a light-emission source in a subject, and shows the horizontal and vertical profile lines along which light-intensity profiles were measured;

25 Figs. 12B and 12C are light-intensity profiles measured along the horizontal and vertical profile lines shown in Fig. 12A;

Fig. 13A shows spectral image plots of luciferase light-emitting sources acquired *in vitro* (solid line), from the subcutaneous site shown in Fig. 13B (squares); and from the lung sites seen in Fig. 13C (diamonds).

30 Figs. 13B and 13C are surface light-intensity images from animals with a subcutaneous light-emitting source (Fig. 13B) and light-emitting sources in the lungs (Fig. 13C).

Detailed Description of the Invention

I. Definitions

Unless otherwise indicated, the terms below are defined as follows:

A "biophotonic function" is a mathematical function that describes a measurable photonic output, such as a light emission profile, spectral intensity distribution, or integrated light intensity, in terms of photonic-source variables, such as source depth, size and shape, number of light-emitting cells, wavelength-dependent scattering and absorption coefficients for specific types of tissue, and spectral characteristics of the light-emitter. The photonic function can be based, for example, on a photon-diffusion model, as discussed below, on a Monte-Carlo simulation, or finite element analysis.

"Turbid tissue" is non-transparent tissue in a subject that has both light scattering and light absorption properties.

"Target region" refers to an internal subsurface region in a subject, such as a subsurface tissue or organ, a solid tumor, or a region of infection, that is (i) localized within the subject, (ii) separated from the surface region of the subject by turbid tissue, and (iii) preferably possesses at least one distinguishable feature, such as a tissue or organ-specific antigen, gene or gene product, either as mRNA or expressed protein, a product resulting from activation, deactivation, or regulation, of a cell, organ, or tissue.

Light-emitting source refers to a source of light emission at the target region. The source may itself emit visible light, such as when the target tissue is genetically modified to produce recombinant light-emitting protein when specific genes are expressed by some activation means. In preferred embodiments, different types of light-generating proteins, such as bioluminescent proteins (e.g., luciferases) and fluorescent proteins (e.g., GFP, dsRed, and YFP) are employed. Exemplary light-emitting sources include prokaryotic and eukaryotic cells transformed with a light-generating protein and administered to the animal subject, as well as light-producing cells which are intrinsically part of an animal made transgenic for a light-generating reporter gene.

Alternatively, the source may include fluorescent or other molecules that can be excited by electromagnetic radiation to emit visible light. For example, a

composition effective to cause a target to emit light may be administered to the subject and allowed to localize at the target location wherein the composition will then emit light, or cause some other moiety in or adjacent the target to emit light. For example, the administered composition may be a compound which activates
5 cells in or adjacent the target, and in-turn, causes such cells to emit light, hence, the localized cells are the target within the subject's body region. In yet other embodiments, infectious cells are administered to a subject, and the progress and locality of the disease are determined. The target, in this embodiment, is the cluster of cells at a given point having a location within the body region of the
10 subject. In other embodiments of the invention, a body region may have multiple targets, and a subject may have multiple body regions, each region potentially having a target.

"Fluorochromes" or "fluorophores" are molecules which are excited at or effectively near an excitation maxima, and emit or fluoresce light at longer
15 wavelengths. The difference between the excitation maxima of a fluorochrome, and the emission maxima of the fluorochrome, is known as a Stokes shift. The greater the Stokes shift for a given fluorochrome, the greater the difference in between the excitation spectrum, or range of wavelengths, which excites the fluorochrome, and the emission spectrum or range of wavelengths of light
20 emitted by the fluorochrome when it fluoresces. Certain embodiments of the invention employ fluorochromes with high Stokes shifts such as Texas Red, rhodamine, Cy3, Cy5, Cy7, and other deep, far red, or near infra-red (NIRF) fluorochromes. Particularly preferred fluorochromes emit light at wavelengths longer than about 600 nanometers. Other preferred fluorochromes will emit light
25 in ranges shorter than 600 nanometers. In particular, variants of "green fluorescent protein" (GFP), each having various emission spectra, are particularly useful because, like luciferase, such proteins may be synthesized by the target or cells adjacent or associated with the target, or which localized at the target, for example, as in an infection. GFPs may further be administered as a
30 conjugate to another protein or biological material that localizes at the target or targets.

In preferred embodiments of the invention, each light-emitting source, under known light-emitting conditions, has a known emission spectrum. Each emission spectrum is preferably constant throughout the detection step. In particular, the relative intensity of light emission at each wavelength of light remains relative to the intensity of light emission at all other wavelengths of light within the emission spectrum of the light-emitting source, as the overall intensity of the spectrum changes. Some embodiments of the invention minimally require at least two distinguishable ranges of wavelengths of emitted light. More preferably, particular embodiments of the invention require that each range of wavelengths of light behave differently, as a function of the distance or depth for which such light travels through the body region, from other ranges as they travel through a selected body region of a subject. For example, the intensity of a first range of wavelengths of light is cut in half for every centimeter of a body region the light travels, whereas a second range of wavelengths of light is cut by three fourth for every centimeter of body region the light travels through. The intensity of light having both ranges of wavelengths, where each range has the same intensity, will diminish by one half at the first range, and three quarters at the second range, after passing through one centimeter of tissue. By comparing the resulting 2:1 ratio of intensities from the initial 1:1 ratio of intensities at tissue depth zero, a determination that the light traveled one centimeter through the tissue can be made.

The invention may be practiced using different light-emitting sources such as different luciferase enzymes. For example, the light emission from "blue" bacterial luciferase (*Photorhabdus luminescens*) expressed on agar was analyzed by a spectrometer that revealed a spectrum centered about 485 nm. A "green" firefly luciferase (*Photinus pyralis*) expressed in PC-3M cells in PBS solution emitted light demonstrating a spectrum centered about 570 nm. A "red" luciferase expressed in PC-3M cells suspended in PBS solution demonstrated a spectrum centered about 620 nm.

II Apparatus and Method

Fig. 1A is a cross-sectional view of an apparatus 20 which may be used in investigating the location and size of a light-emitting source in a subject, in accordance with the invention. The apparatus generally includes a light-tight enclosure or chamber 22 within which light-emission events from the subject can be detected. Light tight box 22 may be constructed as described in co-owned PCT publication number WO 200163247, which is herein incorporated by reference in its entirety. Briefly, the chamber is defined by back and side walls, such as back wall 21, and has a front opening (not shown, which can be opened to provide access to the interior of the chamber, and closed to provide a light-tight seal. Although light tight box 22 was designed for small animals, the technology can also be applied to larger mammals, including humans.

Contained within the chamber is a stage 24 on which the subject is placed and preferably immobilized during light-emission detection. Although many of the examples described herein have to do with a small mammalian subject, typically a mouse or a rat, it will be appreciated that the same principles and methods will apply to other animals as well as human subjects, employing suitable scale-up in chamber size and where necessary, suitable increases in the size and number of light emitters in the target region. The stage in the apparatus shown is designed to be raised and lowered to a selected vertical position within the chamber by a conventional wormscrew mechanism shown generally at 26 and under user control. The stage position within the chamber may be monitored by any of a number of known tracking devices that do not require visible light signals.

The viewing optics includes two general components. The first is a lens assembly 28 that serves to focus light-emission events from the surface of the subject onto the detection screen of a photodetector 32, which is typically a photodetector pixel array of a charged-coupled device that can be operated in a cooled condition to minimize intrinsic noise level. The construction of the lens assembly and its optical coupling to the detector is conventional. One preferred CCD is a model 620 CCD, commercially available from Spectral Instruments.

The second, and optional, component of the optical system is a wavelength filter wheel 30 containing a number of optical bandpass filters designed to block light transmission in all but a selected range of visible-light wavelengths. One standard installation includes 3 filters; a shortpass filter 30a, for wavelengths less than 510 nm (blue light filter), a midpass filter 30b having a bandpass in the 500-570 nm range (green light filter), and a longpass filter for wavelengths greater than 590 nm (red light filter). In some applications, filters can provide more precise bandpass, e.g., every 20 nm. The just-described viewing optics and detector are also referred to herein, collectively, as an optical system.

The apparatus also includes a control unit 34. Various user-controlled settings, such as stage height, orientation, and translation position, bandpass filter, and detector mode are made through a control input pane; 36 in the apparatus.

Fig. 1B shows a more schematic representation of apparatus 20, here shown in a scale for use in imaging a small mammalian subject, such as a mouse 38. The lens assembly is represented here as a single lens 28, the filter wheel at 30, and a CCD detector at 32. The detector output is supplied to a computational unit 40 in the control unit computational unit 34 which includes a processor 40 and a storage unit 42 for data files. The content of the data files, and operation of the computational unit, in response to light-detection signals from the designs from detector 32 will be described below. The control unit is operatively connected to a display/output device 44, such as a computer monitor, for displaying information and images to the user.

The operation of the computational unit can be appreciated from the method of the invention carried out by the apparatus, which will be summarized with respect to Fig. 2, and detailed further below. The first step in the method is to obtain a first measured light-intensity profile. This is done by first localizing a light-emitting source within a subject. This may be done, as noted above, in a variety of ways, including introducing into the subject, a gene effective to produce a light-emitting protein, such as luciferase, in the target region, or localizing a fluorescent compound at the target source, both according to

methods which are known, e.g., as described in co-owned PCT applications WO99US/30078, WO00US/7296, WO99US30080; and WO97US6578, all of which are incorporated herein by reference.

5 The subject so prepared is placed in the light-tight chamber, and light-emission from the subject surface originating from light-emission events at the target region are measured, digitized, and placed in the computational unit, as at 52 in Fig. 2. The computational unit uses this data to generate a spatial profile of light emission, as at 54. This profile could be taken along a selected row and a selected column of detector elements in the detector representing profiles along
10 x (horizontal) and y (vertical) axes in a plane parallel to the stage in Fig. 1A. That is, the profile is a plot of measured intensity values along a selected row and column of detector elements, and represents the distribution of light intensity values that are focused from the subject surface onto the detector array. Exemplary light-intensity values measured from a subject are shown in solid
15 lines in Fig. 3B. Fig 3A shows the emission contours, and Fig. 3B shows the vertical profile, as determined at detector pixels corresponding to those positions.

Turning back now to Fig. 2, the spatial profile or profiles so obtained are matched or fitted with profiles stored in a database 58 of parameter-based
20 photonic functions which are either empirical functions measured from light-emitting sources of known size and depth, or are calculated from photon-diffusion models using depth and size parameters, and optionally, other parameters, as will be described below.

From the optimal curve match, the program can make an initial estimated
25 determination of depth and/or size and/or brightness, preferably all three, of the light-emitting source. According to a feature of the invention, the method is now carried out in a way that the depth and size determinations are refined by additional data that may be in the form of additional light-intensity data, and/or additional modeling data. For example, and as indicated in Fig. 2, the data may
30 be in the form of:

(a) 2nd-view data, as at 62, meaning light-intensity data, typically spatial profile data, obtained by viewing the subject from a second view angle.

Typically, the subject in the apparatus is tilted with respect to the optical system so that light-emission is obtained from a second region of the subject surface;

(b) Spectral data, as at 64, meaning light-intensity data obtained at one or more bandpass ranges, e.g., in a blue-light, green-light, or red-light spectral range;

(c) Transmittance data, as at 66, meaning predetermined-light intensity values obtained typically as a function of wavelength at selected locations in a subject that approximates the subject of interest;

(d) Tissue properties data, as at 68, meaning predetermined values of tissue parameters, such as reduced scattering coefficient μ'_s , absorption coefficient μ_a , or effective coefficient μ_{eff} corresponding to the subject tissue through which light is diffusing. This data is used, for example, to refine the spatial profile curves generated by a diffusion model;

(e) Simulation data, as at 70, meaning predetermined light-intensity values, typically spatial profile data, obtained by placing a light-source of known intensity and size at selected locations in a model representing the subject target region and adjacent surface, or in an actual subject that approximates the subject; and

(f) Total intensity data, as at 72, meaning total light intensity summed or integrated over an entire spatial profile or an entire detector array.

The program run by the computation device receives the additional information at 60, and uses the information to refine the depth and size determination of the light-emitting source, at 74, as will be considered below. Finally, the refined determination of source depth, size, and optionally, shape and number of cells in the light-emitting source, is displayed to the user, either in the form of data and/or one or more subject images showing the location and intensity of the light-emitting source.

(g) Absolute calibrated light intensity, as at 73. Calibration to absolute intensity allows one to convert counts/sec/pixel in the CCD to radiance photons/s/cm²/sr (sr=steradians). Fig. 8A illustrates an optical assembly used in the calibration method. The assembly includes, in addition to a CCD detector 102, lens 104 (representing a lens assembly, as above), a bandpass

filter 106 and a low-light-level integrating sphere, such as a OL Series 425 sphere available from Optronic Laboratories, which acts as a source of a known light intensity. The known radiance from the sphere, measured in photons/sec/cm²/sr, allows one to calculate the calibration factor that relates the two numbers.

Fig. 8B illustrates how absolute measured intensity is used to calculate total number of cells in a light-emitting source. It is assumed that each cell will produce a photon flux ϕ_c photons/sec/cell. The radiance from a light-emitting source 110 in a subject 112, as measured by detector 102, measures the number of photons/s/cm²/sr. The measured radiance is then integrated over a region of interest to convert to a source flux ϕ_s photons/sec. From the known flux values, the number of cells in the light-emitting source is readily calculated as ϕ_s/ϕ_c .

III. Light-emission data and parameters

This section considers various types of light-emission data that may be collected in practicing the invention, and discusses how the data may be used in photon-diffusion modeling, and/or for determining depth or size of a light-emitting source based on curve matching with a model diffusion curve.

Light-intensity spatial profile. The basic light-emission data that is determined is a light-intensity spatial profile which has been described above, with reference to Fig. 3B. A measured light-intensity spatial profile shows the spatial distribution of light intensity emitted from the surface region of subject. The light-intensity spatial profile may be obtained along a single line, along 2 or more lines (e.g., along the x and y directions with respect to the stage supporting the subject). The entire 2D surface distribution of light may also be used. A modeled spatial profile represents the predicted light-intensity spatial distribution calculated from a model of photon diffusion through a turbid tissue. By matching a measured profile with a modeled profile, an approximate depth and/or size of light-emitting source can be determined.

Total light intensity. Total light intensity is the total light intensity summed or integrated over all of the detector elements in the detector. Total light

intensity can be used as a further constraint on the fitting model used to determine source, location, and brightness.

Absolute calibrated light intensity. As described above with reference to Fig. 8B, the absolute light-intensity flux can be used to measure total flux from the light-emitting source. Assuming that this source is made up of cells which each emit an average cell flux ϕ_c , the total number of light-emitting cells making up the source can be determined. This determination, in turn, can be used to refine the total source mass or volume determination, based on a known or estimated cell mass ratio for the tissue source.

Spectral data. Spectral data is light-intensity data collected at particular wavelength ranges, e.g., the blue-light, green-light, and red-light wavelength ranges noted above. Because light is absorbed preferentially at shorter wavelengths by subject tissue, and particularly by hemoglobin in subject tissue, the relative intensities of light at different wavelengths, can provide information about source depth and can be used in producing refined wavelength-dependent spatial profiles, based on wavelength-dependent tissue scattering and absorption coefficients.

Figs. 4A and 4B depict the differential effect distance or depth has upon light that travels through the tissue of the body region, at different wavelengths or ranges of wavelengths. The graph shows increasing light intensity on the y-axis and increasing light wavelength on the x-axis. The solid line in Fig. 4A represents the *in vitro* emission spectra for the light-emitting source that correlates to a zero-depth emission spectrum which comprises a broad single peak centered about 495 nm. The broad dashed line represents the emission spectrum of the light-emitting source after it has passed through about one millimeter of tissue (*in vivo*). At about 600 nm, the one millimeter depth emission spectrum develops a small peak, as the once large peak at about 495 nm begins to diminish in relative intensity. The intermediate dashed line represents the emission spectrum of the light-emitting source after it has passed through about two millimeters of tissue (*in vivo*). The 495 nm peak becomes lesser in intensity, while the 600 nm peak increases and becomes more pronounced. Lastly, the dotted line represents the emission spectrum of the light-emitting source after it

has passed through about four millimeters of tissue (*in vivo*) where the 495 nm peak has been further reduced.

Fig. 4B represents the emission spectrum at four millimeters depth of tissue scaled against the zero tissue depth emission spectra. The 600 nm peak is prominent over the once prominent 495 nm peak. In this example, it is the ratio of the 600 nm peak to the 495 nm peak that provides information about the depth of the light-emitting source.

Preferred methods of the invention employ self-scaling computations so that the depth of the light-emitting source may be determined independent of its overall intensity. To achieve this end, functions are formulated that utilize ratios of two or more ranges of wavelengths, where each range of wavelengths travels through tissue in a body region at different rates as a function of depth of the target from the surface of the body region.

Through-body transmittance. Through-body transmittance is measured by placing a know-intensity light-source at a selected location against or inside a subject, and measuring the light-intensity that is transmitted through the subject on multiple surfaces. The transmittance will have a strong spectral component, owing to the strong spectral dependence of the scattering and absorption coefficients of different tissues (see below). Typically, transmittance values are pre-determined using a model subject, by measuring total transmittance at various selected locations and at each of a number of spectral ranges.

Fig. 5 shows a top view of a subject having a plurality of body regions indicated by numbers 1-16. In this example, the subject is an immobilized mouse, and each body region corresponds to pre-determined regions of interest. Prior to depth determinations by the practice of the methods of the invention, the subject is optically analyzed to develop a data file of optical characteristics for each body region. In this example, each body region is placed between a spectral light source and a spectral detector. Light transmission spectra are obtained for each body region. The result is analogous to placing each body region in a spectrophotometer cuvette and measuring the spectral profile of each region at different wavelengths and wavelength ranges, to create a data file of how light transmits through different body regions, and the tissues within such

regions. The device of Fig. 1 may be used to develop similar data for particular ranges of wavelengths provided the device is adapted to trans-illuminate, with respect to the detector, the subject at each body region tested. Thus, the device of Fig. 1 becomes, in essence, a whole animal spectrophotometer.

5 Fig. 6A and 6B show the empirical spectral data gathered from the spectral analysis described immediately above. As is described below in more detail, this data, either as whole spectra, or particular ranges within each spectrum, can be used to create the data files for comparison with detected intensity information, or for developing functions to compute target depth.

10 In a more detailed approach, the apparatus provides a whole body scanner for scanning an entire subject, or portions thereof, which further comprises a scanning two-color laser for scanning the body of a subject. The scanner moves through an arc laser light of at least two colors, either simultaneously, or sequentially. As the laser light beams upward, it penetrates
15 the body of a subject placed within a rotational tube. The detector placed opposite the laser light source is adapted to receive and measure the laser light as it moves through its arc path.

Tissue optical properties. There are two important optical properties of subject tissue used in photon diffusion modeling. The first is the absorption
20 coefficient μ_a of the tissue, which is related to the fraction of incident light that is absorbed per unit pathlength. As seen in Figs. 7A and 7B, which plot absorption coefficients for a variety of tissue types over the visible spectrum, the absorption coefficient is highly dependent on the nature of the tissue, and the wavelength of light, with each tissue showing a peak absorption in the 500-600 nm range. The
25 relatively low absorption coefficient above about 600 nm is consistent with the spectral data seen in Fig. 13A, and with the total transmittance data shown in Figs. 6A and 6B.

The second important optical property is the reduced scattering coefficient μ'_s of the tissue, which is the fractional decrease in intensity per unit length of
30 penetration of light due to large angle light scattering. As seen in Figs. 7C and 7D, which plot reduced scattering coefficient for the same tissues over the visible spectrum, scattering effects can be relatively different in different tissues. In

general, the reduced scattering coefficient decreases somewhat at longer wavelengths.

A double integrating sphere (DIS) system is a widely used tool to measure the optical properties of tissue or any turbid medium since it conveniently
5 measures the diffuse reflectance, R_d , and the diffuse transmittance, T_d , simultaneously. Fig 6C illustrates the double integrating sphere apparatus 74 used to measure these values in order to obtain optical properties such as the absorption coefficient, μ_a , and the reduced scattering coefficient, μ'_s (Prahl, S.A.,
10 *et al.*, *Applied Optics* **32**:559-568 (1993); Pickering, J.W., *et al.*, *Applied Optics* **32**:399-410 (1993)). The sample 76 is placed between the two integrating spheres 78 and 80, with internal baffles 82 positioned to prevent measurement of directly reflected or transmitted light. The spheres are coated with a highly reflective material to minimize absorption of light. Light is detected with detectors 84 and preferably analyzed using a computer 86. Sample illumination
15 between 400-1000nm is achieved using an arc lamp connected to a monochromator 88, via a fiber optic cable 90 and lens 92.

From diffuse reflectance, R_d , and diffuse transmittance, T_d , values, the absorption and the reduced scattering coefficients are obtained using the inverse adding-doubling program (<http://omlc.ogi.edu/software/iad/index.html>). This
20 program is a numerical solution to the one speed radiative transport equation, which describes light propagation at steady state in a scattering medium (Prahl, S.A., *et al.*, *Applied Optics* **32**:559-568 (1993)). The program is an iterative process, which estimates the reflectance and transmittance from a set of optical parameters until the calculated reflectance and transmittance match the
25 measured values.

A characteristic of the DIS system is the fact that tissues need to be extracted before the measurement. Therefore, it is desirable to maintain tissue viability under measurement conditions. Vascular drainage of hemoglobin and tissue hydration need to be considered, especially at wavelengths where
30 hemoglobin and water absorption are high. Partly in view of the foregoing, a

preferred method of measuring tissue optical properties is a non-invasive *in vivo* measurement, as follows.

A radial fiber probe similar to that described by Bevilacqua (Bevilacqua, F., *et al.*, *Applied Optics* **38**:4939-4950 (1999)) may be used to measure optical properties non-invasively or with minimal invasiveness *in vivo*. Figure 6D illustrates an experimental set up of a radial fiber probe 94. Fiber probe 94 includes an illumination source 95, at least one illumination fiber 96 and several (e.g., six) detection fibers 97 generally within a 1-2 mm distance. The probe is then placed perpendicularly on a tissue of interest 99, preferably with minimal contact pressure. The output of the detection fibers 97 is fed into a set of corresponding detectors 98. The optical properties are spatially resolved from intensity versus radial distance from the source. At small source-to-detector separations, simple analytical models such as the diffusion approximations are not well suited to describe the system therefore Monte Carlo methods are typically used to analyze the data.

IV. Photon transport models

This section considers photon-transport models for simulating photon emission at the surface of a body from a light source below the surface and moving through a turbid medium. In particular, it is desired to generate simulated light-intensity spatial profiles based on (i) depth of the light-emitting source, (ii) size of the light-emitting source, and (iii) light-absorption and light-scattering coefficients. Since the light-absorption and light-scattering coefficients are dependent both on wavelength and on the nature of the tissue through which the photons diffuse, the model may be refined to specifically consider the nature of the tissue and the spatial profiles at selected wavelengths.

The photon-diffusion model employed herein makes a number of simplifying assumptions for purposes of generating an initial light-intensity spatial profile from which initial source-depth and source-size information can be generated, as will be considered in Section V. The model may then be expanded by considering additional tissue-dependent and/or wavelength dependent information to refine the spatial profile, for purposes of improving the

curve fit with a measured spatial profile. Alternatively, the initial depth and size information can be refined by other types of curve or data matching. Both approaches for refining the initial depth/size approximation will be considered in Section V below.

Fig. 9A shows a photon path as it diffuses through a turbid tissue formed of cells, such as cell 111 containing organelles 113. As seen, the cell size, typically in the 20-30 micron size range, is large relative to the wavelength λ of light, which is between about 0.4 and 0.6 microns. Scattering is due to abrupt discontinuities ($\ll \lambda$) at membranes and is characterized by an inverse length scattering coefficient μ_s of about 10-20 mm⁻¹. The scattering anisotropy g is about 0.9, which gives a reduced scattering coefficient $\mu'_s = \mu_s (1-g)$, or about 2 mm⁻¹. The absorption coefficient $\mu_a(\lambda)$ is between 0.01 and 1 mm⁻¹, and as seen above, has a strong wavelength dependence. The absorption and scattering coefficients are wavelength-dependent, but for $\lambda > \sim 600$ nm, it is generally true that $\mu_a \ll \mu'_s$ in tissue.

A quantitative description of transport of light from a target site within a body to a light detector located adjacent a body surface may be achieved by a solution of a radiative transfer diffusion equation of light with appropriate boundary conditions (e.g., R.C. Haskell; et al in "Boundary conditions for the diffusion equation in radiative transfer", J. Optical Soc Am., 11A:2727 (1994)). One approach, for example, uses an extrapolated boundary condition shown schematically in Fig. 9B in which optical fluence vanishes at a planar surface that is displaced from the physical boundary at a distance z_b that takes account of the Fresnel reflection of light at the physical boundary.

As a first approximation, the body in which the light-emitting source is located is represented as a semi-infinite, homogenous turbid medium that is both scattering and absorbing. The approximation is illustrated in Fig. 9B which shows a light-emitting point source 114 in slab 116 a distance d below the slab surface. The diffusion equation is

$$D\nabla^2\phi(r) = \mu_a\phi(r) - S(r) \quad (1)$$

and Fick's law is

$$j(r) = -D \nabla \phi(r) \quad (2)$$

where Φ is the isotropic fluence (watts/m²), j is the small direction flux (watts/m²), S is the power density (watts/m³), r is the radius, and

5

$$D = \frac{1}{3[(1-g)\mu_s + \mu_a]} \quad (3)$$

The Green's function solution for a point source P (watts) is

$$\phi(r) = \frac{P}{4\pi D r} \exp(-\mu_{eff} r) \quad (4)$$

10

where $\mu_{eff} = \sqrt{3\mu_a(\mu'_s + \mu_a)}$ and $\mu'_s = (1-g)\mu_s$.

The solution to the diffusion equation in slab geometry using the extrapolated boundary condition is obtained by summing contributions from the source 114 plus image source 118 (as shown in Fig. 9B), resulting in the following equation for radiance at the surface (viewing perpendicular to the surface) for a point source P (watts) at a depth d .

15

$$L_{z=0} = \frac{1}{4\pi} \left(\frac{P}{4\pi D} \right) \left\{ \begin{array}{l} \frac{\exp(-\mu_{eff} r_1)}{r_1} - \frac{\exp(-\mu_{eff} r_2)}{r_2} \\ + 3D \left[\frac{d}{r_1^2} \left(\mu_{eff} + \frac{1}{r_1} \right) \exp(-\mu_{eff} r_1) + \frac{d+2z_b}{r_2^2} \right. \\ \left. \times \left(\mu_{eff} + \frac{1}{r_2} \right) \exp(-\mu_{eff} r_2) \right] \end{array} \right\} \quad (5)$$

20

where $r_1 = \sqrt{\rho^2 + d^2}$, $r_2 = \sqrt{\rho^2 + (d+2z_b)^2}$ ρ is the radius on the slab surface and

$$z_b = \frac{1 + R_{eff}}{1 - R_{eff}} \frac{2}{3(\mu_a + \mu'_s)} \quad (6)$$

and R_{eff} is the effective reflection coefficient which is about 0.43 for tissue.

Plotting surface radiance as a function of ρ gives the plot shown in the solid line in Fig. 10B, which has the same derivation as the light-intensity profile plot shown in dotted lines in Fig. 3B.

5 The spatial profile curve for a light-emitting point in a turbid medium was also calculated using a much more computationally intensive Monte Carlo simulation, which follows each photon through a random walk, as illustrated in Fig. 10A. The Monte Carlo simulation, shown in "+" symbols in Fig. 10B closely matches the spatial profile calculated from the diffusion equation.

10 To illustrate how the diffusion equation may be used to model photon diffusion through various tissues and at various depths, the spatial profile was calculated for various values of μ_{eff} , corresponding to μ_a and μ'_s values of 2.0 and 20 cm^{-1} , respectively, for $\mu_{\text{eff}} = 11 \text{ cm}^{-1}$; 0.4 and 10 cm^{-1} , respectively, for $\mu_{\text{eff}} = 3.5 \text{ cm}^{-1}$, and 0.05 and 5 cm^{-1} , respectively, for $\mu_{\text{eff}} = 0.87 \text{ cm}^{-1}$. The three values of μ_a correspond roughly to tissue absorption for blue, green, and red
15 wavelengths.

The plots in Figs. 11A and 11B show that intensity decreases and spot width increases with increasing depth, and that large values of μ_{eff} result in large attenuation of peak intensity with narrower spot width.

20 V. Determining Depth and Size information

In practicing the method of the invention, the subject is initially treated to localize light-emitting molecules at a target source, as detailed above. The subject is then placed at a selected location within the light-tight chamber of apparatus 20 (appropriately scaled for subject size) and the optical system is
25 adjusted to measure light emission events from the source at the surface region of the subject between the light-emitting source and the detection optics. With the subject immobilized at a selected optical perspective, a light-intensity spatial profile of surface light emission is obtained, also as described above. Fig. 3A is a surface map of measured light intensity from a subsurface light source, which
30 was subsequently used to generate the spatial profile shown in Fig. 3B.

The spatial profile(s) are then fitted with parameter-based biophotonic functions that relate light-emission intensity to depth of a light-emitting source

(and in some cases, source size) to obtain an initial determination of source depth (and in some case, source size). One preferred biophotonic function is that derived from the simplified diffusion model discussed in Section IV above, in which light intensity from a point source or source having a defined spherical or ellipsoidal volume is calculated as a function of source depth, fixed scattering and absorption coefficients, and surface distance r from the source.

Conventional non-linear least-squares curve fitting techniques, such as Levenberg-Marquardt ("Numerical Recipes in C", Press *et al.*, eds, Cambridge Press, NY, 1989). are suitable for the curve fitting. Curve fitting can be done using a single 1-dimensional ("1-D") profile, as shown in Fig. 3, a plurality of such profiles, or the entire 2-D spatial distribution (e.g., as shown in Fig. 12A). Curve-fitting calculations to the data shown in Fig. 3B (dashed line) indicate a depth of 2.7 mm, which compares well with the estimated actual depth of 2.2 mm.

Figs. 12A-12C give another example of how depth and size information can be determined from spatial profiles. The light-emitting source here is a subcutaneous elliptical tumor having actual dimensions in the horizontal and vertical directions of 2.7 mm and 3.2 mm, respectively, and a tumor thickness of 1.5 mm. The horizontal and vertical profiles were (Figs. 12B and 12C, respectively) were fitted with curves generated for an ellipsoid light-emitting source having horizontal and vertical dimensions of 1.3 and 2.4 respectively, a thickness of 1.5 mm, and a depth of 0.4 mm.

The source depth (and optionally, source size) determination from the initial curve fitting can be refined, and source size approximated, by employing additional data whose effect is to refine the parameters of the biophotonic function. The nature of the additional data, and the manner in which it refines the parameters, e.g., depth and size, of the biophotonic function, providing refined depth and source-size information, will now be considered for each of the information types outlined in Section II above, with respect to Fig. 2.

A. 2nd-view information. To obtain 2nd-view data, the subject is rotated with respect to the viewing and detection optics for viewing the light-emitting source from another perspective. From this second perspective, a second light intensity profile is obtained, providing a second depth determination with respect

to a second subject surface region. By determining the intersection of the source depths at two different surface locations, a more accurate depth and/or source size can be determined. The more views that are obtained, the more accurately depth and source size can be determined.

5 B. Spectral data. A target's depth within a body region may be determined, or the target depth refined, by (i) causing the target to emit light at two or more wavelength ranges, and (ii) comparing the differences in each range's light transmission through the body region, at a surface of the body region, provided that the light in each wavelength range transmits through the
10 body region differently from the light in other wavelength ranges as a function of depth.

Typical spectral curves (representing total light intensity at the indicated wavelengths) as a function of source depth are shown in Fig. 4A, discussed above. As seen particularly in Fig. 4B (where the peaks are scaled to the *in vitro* spectral curve), the ratio of peak height at above 600 nm, e.g., 620 nm, to that
15 spectral curve), the ratio of peak height at above 600 nm, e.g., 620 nm, to that the *in vitro* (zero-depth) peak height, e.g., around 500 nm, increases dramatically as a function of depth. Thus, by generating a spectral curve and determining the ratio of peak heights, typically above and below 600 nm, e.g., 620nm: 500 nm, accurate depth information based on the wavelength-dependent loss in intensity
20 in turbid tissue, can be determined.

A useful formula for determining approximate depth from spectral measurements can be derived from Eq. 4 as follows:

$$d \approx \frac{\ln(\phi_1 D_1 / \phi_2 D_2)}{\mu_{eff2} - \mu_{eff1}} \quad (7)$$

25

where d represents depth, ϕ represents the measured light intensity, μ_{eff} represents the empirically determined effective coefficient of attenuation, and D is the empirically determined diffusion coefficient. The subscripts 1 and 2 in the above formula refer to two separate wavelengths at which measurements are
30 made.

Using the above formula, depth may be determined from two or more light intensity measurements at different wavelengths or ranges of wavelengths. In one embodiment, the depth may be obtained by executing the following series of steps: (1) Image the bioluminescent cells *in vitro* as well as *in vivo* (in the animal) at two wavelengths; (2) Quantify the images by measuring, e.g., the peak intensity or the average (integrated) intensity for each image; (3) Calculate the ratio of the *in vivo* image data to the *in vitro* image data at each wavelength; and (4) Calculate the depth using Equation 7 and an effective scattering coefficient μ_{eff} obtained, e.g., from tissue property measurements. An application of this approach is illustrated in Example 1.

In one embodiment, it is preferable to have a significantly different value for each μ_{eff} . Animal tissue provides such a difference in μ_{eff} , due largely to the presence of hemoglobin, which has a large absorption peak just below 600 nm, and relatively low absorption above 600 nm.

Alternatively, the additional spectral data may include one or more spatial profiles taken at one or more selected wavelengths or wavelength ranges. The profile(s) are then compared with model intensity functions generated, for example, from a photon diffusion model above, using wavelength-specific values for the absorption, scattering, and/or effective coefficients, as described above with respect to fig. 3B.

In still another application, intensity values can be measured at discrete wavelengths, spaced every 20 nm. The spectral plots in Fig. 13A show green luciferase in cells in PBS solution, and spectral curves for green luciferase localized subcutaneously at a site indicated in Fig. 13B and in the lungs as shown in Fig. 13C. All the curves are normalized to a value of 1 at 700 nm. The ratio of intensities at 560nm to 620nm is indicative of depth.

As noted above, a related aspect of the invention provides a method of determining the depth of a light-emitting source in a subject. In practicing the method, the light emission intensity from the subject at two or more different wavelengths between about 400 and about 1000 nm. The depth of the light-emitting source is determined using the measured light-emission intensities, and

information related to the optical properties of the subject, e.g., coefficients of attenuation and diffusion in the subject.

Information relating to the optical properties, .e.g., coefficients of attenuation and diffusion in the subject may be obtained by direct measurement
5 of the coefficients in material that is the same or similar to that of the subject. This information, combined with measured intensities at two wavelengths *in vitro* (zero depth) and *in vivo* (the depth to be determined) can be applied to Equation 7 to determine the depth of the light source, as described above, and as illustrated in Example 1.

10 Alternatively, optical-properties information may be obtained indirectly, by determining, at two or more different wavelengths, light intensities from a light-emitting source located at each of a two or more of a plurality of depths in tissue or material corresponding to that of the subject. The desired depth
15 determination is then made by matching the measured light intensities, e.g., the ratio of the intensities at the different wavelengths, with the light intensities determined at each of the plurality of depths. In a more specific approach, the spectral profile of light intensities from the light-emitting source may be compared (matched) with each of a plurality of spectral profiles of light intensities from a light-emitting source at each of a plurality of depths.

20 C. Through-body transmittance data. Through-body transmittance data is obtained as described above, and illustrated in Figs. 5, 6A and 6B for a small-animal subject. As noted above, the information provides a wavelength
dependent light-transmission value through a selected tissue or tissues of a known thickness. Predetermined transmittance data from a number of selected
25 locations in a subject can be used to estimate average whole-body scattering and absorption coefficients at the different subject locations, for purposes of refining the modeled spatial profile, either over the entire visible spectrum, or at selected wavelengths, used for curve fitting to the measured spatial profile.

D. Tissue properties data. The tissues property data, such as that shown
30 in Figs. 7A-7D, typically includes wavelength dependent absorption and scattering coefficients for each of the major body tissues. This data is used, for example, in the above model of photon diffusion, to refine the spatial profiles that

would be produced by light transmission through a given tissue, at a given wavelength, typically in the red wavelength. Thus, for example, if the light-emitting source in the subject is muscle, and the spatial profile is taken in the red wavelength, a refined spatial profile generated with the tissue-specific and wavelength-specific absorption coefficient will provide a refined spatial profile curve for curve fitting with the measured curve.

E. Simulation data. In another embodiment, a test point or otherwise known-shape light-emitting source may be introduced into a block or slab that simulates turbid tissue. The block can be prepared with various scattering and diffusion coefficients, and various shapes to simulate light-emitting source conditions in a subject. Upon placement of the test point, spectral profiles may be taken from the outer surface of the body region where the test point was placed. This data then is correlated with the actual depth and location of the test point. By moving the test point from point to point within the block, a series of spectral measurements can be made. From this series of spectral measurements, a data file can be assembled to model the spectral responses of different regions within the subject.

F. Integrated light intensity

Summed or integrated light-intensity, as noted above, refers to the light-intensity summed over all or some defined area of the detector array. The integrated light intensity can be compared with the integral of Equation 5 to provide yet another estimate of source depth and brightness. This information can be used in conjunction with the profile information. The integrated light intensity can also be calculated for multiple wavelengths.

G. Calibrated-intensity data. In the absence of calibrated intensity data, intensity measurements may vary from camera to camera, and measured values will depend on variable such as field of view, binning, time, and f-stop.

Absolute calibrated intensity (Section III above), allows one to refine the depth-of-source determination, based on curve fitting to true peak value, and to estimate the number of bioluminescent cells in tissue, as discussed in Section III above.

In still other embodiments, the invention provides for the integration of other imaging data with the intensity and spatial distribution data to produce greater detail three-dimensional maps of the subject and target located therein. For example, compiled differential light transmission data, such as disclosed
5 above, may be interlaced with coordinate systems derived from other three dimensional imaging systems such as stacked slices of MRI images forming a three dimensional digitized "image" or coordinate system. Such a coordinate system may then be used to better illustrate the anatomy of the subject for which a target is being identified. In the case where the subject is a laboratory animal
10 such as a rat, such animals are highly uniform in structure from one animal to the next for a given strain. Consequently, a coordinate data, spectral data, and spatial intensity pattern files may be developed by a commercial vendor, and sold for use with simplified detection units such as shown in Fig. 1. If model three-dimensional information is supplied as a data file, then user units do not
15 need to be equipped for three-dimensional scanning and mapping. The two-dimensional images contemplated in the methods and apparatuses disclosed above may be combined with the three-dimensional data files provided by the vendor to yield complete three dimensional information about the size, shape, depth, topology, and location of a target within the subject.

20 The following examples illustrate, but in no way are intended to limit the present invention.

EXAMPLE 1

CALCULATING DEPTH OF A LIGHT-EMITTING OBJECT USING TWO-WAVELENGTH 25 SPECTRAL IMAGES

The spectral information shown in Figure 13A was used to calculate the depth of luciferase-labeled cells in an animal that received a sub-cutaneous
30 injection of the cells (Fig. 13B) and an animal having labeled cells in its lungs (Fig. 13C). The analysis was performed using data at wavelengths equal to 600 nm and 640 nm using the following steps: (i) the bioluminescent cells were

imaged *in vitro* as well as *in vivo* at 600 nm and 640 nm; (ii) the images were quantified by measuring the average intensity for each image; (iii) the ratio of the *in vivo* image data to the *in vitro* image data were determined at each wavelength; and (iv) the depth was calculated using Equation 7 and an average effective scattering coefficient μ_{eff} estimated from the absorption and scattering coefficients in Fig 7.

For the subcutaneous cells, the ratios of *in vivo* to *in vitro* intensities (relative intensities, or ϕ) are 0.35 and 0.75 at 600 nm and 640 nm, respectively. For the lung signals, these same ratios are 0.05 and 0.47. Using $\mu_a=0.25 \text{ mm}^{-1}$ and $\mu_s'=1.0 \text{ mm}^{-1}$ at 600 nm and $\mu_a=0.05 \text{ mm}^{-1}$ and $\mu_s'=1.0 \text{ mm}^{-1}$ at 640 nm results in $\mu_{\text{eff}}=0.97 \text{ mm}^{-1}$ at 600 nm and 0.4 mm^{-1} at 640nm. The values of the diffuse coefficient D are 0.27 mm and 0.32 mm at 600 nm and 640 nm respectively. Substituting these numbers into Equation 6, reproduced below,

$$d \approx \frac{\ln(\phi_1 D_1 / \phi_2 D_2)}{\mu_{\text{eff}2} - \mu_{\text{eff}1}}$$

(in this case, subscript 1 refers to 600 nm and subscript 2 refers to 640 nm), results in $d = 1.6 \text{ mm}$ and $d = 4.0 \text{ mm}$ for the subcutaneous and lung depths, respectively.

Although the invention has been described with respect to particular embodiments and applications, it will be appreciated that various changes and modification can be made without departing from the invention.

IT IS CLAIMED:

1. A method of investigating the location and size of a light-emitting source in a subject, comprising:

5 (a) obtaining a first measured light intensity profile constructed by measuring, from a first perspective with a photodetector device, photons which (i) originate from the light-emitting source, (ii) travel through turbid biological tissue of the subject, and (iii) are emitted from a first surface region of interest of the subject;

10 (b) fitting said first measured light intensity profile with a parameter-based biophotonic function; and

(c) refining the parameters of the biophotonic function, using data other than said first measured light intensity profile, to obtain an approximate depth and size of the source in the subject.

15

2. The method of claim 1, wherein said refining includes using data relating to the wavelength of photons emitted from the surface of the subject.

3. The method of claim 2, for use in investigating the depth of the light-emitting source in the subject, wherein said refining includes (a) measuring light emission intensity from the subject at two or more different wavelengths between about 400 and about 1000 nm, (b) obtaining information related to optical properties in the subject's tissue, and (c) using the measured light intensities and the obtained information to refine the depth of the light-emitting source.

25

4. The method of claim 3, wherein the steps (a), (b), and (c) above are carried out by a protocol selected from the group consisting of:

A. step (b) includes measuring the coefficients of attenuation and diffusion in tissue or material corresponding to that of the subject. and step (c) includes using said coefficients in a formula to estimate depth of said source;

30

B. step (b) is carried out by determining, at two or more different wavelengths, light intensities from by a light-emitting source located at each of a

plurality of depths in tissue or material corresponding to that of the subject, and step (c) is carried out by matching the measured light intensities with the light intensities determined at each of the plurality of depths.

5 C. step (a) includes measuring a spectral profile of light intensities from the light-emitting source, step (b) is carried out by determining spectral profiles of light intensities from a light-emitting source at each of a plurality of depths, and step (c) is carried out by matching the measured profile with the determined profiles, to identify a best curve fit; and

10 D. step (a) includes measuring the light-intensity profile at a different wavelength.

5. The method of claim 1, wherein said refining includes using data measured from said subject.

15 6. The method of claim 5, wherein said refining includes using data obtained from a second measured light intensity profile, said second profile being constructed by measuring, from a second perspective with said photodetector device, photons which (i) originate from the light-emitting source, (ii) travel through turbid biological tissue of the subject, and (iii) are emitted from
20 a second surface region of interest of the subject

7. The method of claim 5, wherein obtaining said data includes the steps of (i) measuring total light intensity from the first surface region of interest, (ii) estimating tissue-region depth, size, and brightness by comparing the measured
25 light-intensity values with total radiance values generated as a function of depth of a point-source light emitters, wherein the total radiance values are generated from a model of photon-diffusion from a light source of defined size, shape, and/or depth below a body surface.

30 8. The method of claim 1, wherein said refining includes using data obtained from a computer simulation of the diffusion of light from a light-emitting source in a turbid medium.

9. The method of claim 8, wherein said computer simulation is a photon diffusion model.

10. The method of claim 9, wherein said refining comprises (i) generating
5 a plurality of theoretical light-intensity profiles, based on a model of photon
diffusion from a light-emitting source located at one of a plurality of depths, and
having one of a plurality of sizes and shapes, through a turbid medium having
absorption and scattering properties similar to those of biological tissue, (ii)
10 comparing the quality of fit between each of said plurality of theoretical light-
intensity profiles and said first measured light intensity profile, (iii) selecting the
theoretical light intensity profile which provides to the first measured light
intensity profile, and (iv) obtaining an approximate depth, shape and brightness
of the source in the subject using parameters from the theoretical light intensity
profile selected in (iii).

15

11. The method of claim 10, wherein comparing the quality of fit is done
using a least squares algorithm.

20

12. The method of claim 10, wherein comparing the quality of fit is done
using genetic algorithms.

25

13. The method of claim 10, wherein said generating includes employing
in a photon-scattering model, one or more predetermined tissue-specific light-
scattering coefficients corresponding to tissue through which said photons travel.

14. The method of claim 1, wherein said light-emitting source is a
luminescent moiety.

15. The method of claim 1, wherein said light-emitting source is a
30 fluorescent moiety.

16. The method of claim 1, wherein said light-emitting source is administered to the subject and binds to a selected target in the subject prior to said measuring.

5 17. The method of claim 1, wherein said light-emitting source is a light-generating protein.

18. The method of claim 17, wherein said light-generating protein is expressed by biological cells of said subject.

10

19. The method of claim 17, wherein said light-generating protein is expressed by biological cells administered to said subject.

20. The method of claim 1, further comprising generating a visual
15 representation of said light-emitting source, using the approximate depth and shape of the source in the subject, and superimposing said visual representation onto an image of the subject.

21. The method of claim 20, wherein said image is a two-dimensional
20 image.

22. The method of claim 20, wherein said image is a three-dimensional image.

25 23. Apparatus for use in investigating the location and size of a light-emitting source in a subject, comprising:

 a light-tight enclosure within which light-emission events from the subject can be detected,

 an optical system contained within the enclosure for use in obtaining a
30 first light intensity profile constructed by measuring, from a first perspective within the enclosure, photons which (i) originate from the light-emitting source,

(ii) travel through turbid biological tissue of the subject, and (iii) are emitted from a first surface region of interest of the subject; and

a computational unit operatively connected to the photo-detector for (i) fitting said first measured light intensity profile with a parameter-based biophotonic function; and (ii) refining the parameters of the biophotonic function, using data other than said first measured light intensity profile, to generate an approximate depth, shape, and brightness of the source in the subject.

24. The apparatus of claim 23, wherein the optical system includes a charged-coupled-device (CCD) that can be operated in a cooled condition, and a lens for focusing light onto the CCD.

25. The apparatus of claim 24, wherein the optical system is designed to detect photons emitted from a plurality of different selected surface regions of interest of the subject.

26. The apparatus of claim 23, wherein the optical system includes one or more wavelength filters for transmitting photons within different selected wavelength ranges, for use in measuring photon-emission intensities within two or more different selected wavelength ranges between about 400-800 nm, and the computation unit is operable to carry out at least one of the parameter-refinement operations:

(i) determining the relative light intensities measured at the different wavelengths, and comparing the determined relative light intensities with known relative signal intensities at said different wavelengths, as a function of tissue depth; and

(ii) comparing the measured spectrum with a plurality of spectra measured from light-emitting sources placed at various depths within tissue, and determining the depth of the light-emitting target region from matching the measured spectrum with the known spectra.

27. The apparatus of claim 23, wherein said computational unit includes a data file containing predetermined spatial distribution information relating the spatial distribution of light emitted within the selected wavelength ranges as a function of size, shape, and/or depth of the a model target's light emission within
5 a model body region of a model subject, and the unit functions in carrying out task (i) to compare spectral characteristics of the first light-intensity profile with those contained in the database.

28. The apparatus of claim 23, wherein the computational unit is operable
10 to integrate the intensity pattern image into a single intensity value, and to estimate source size by integrated light intensity values generated as a function of a light-emitting source of a particular size and shape.

29. The apparatus of claim 23, wherein said computational unit includes
15 a database containing a plurality of theoretical light-intensity profiles, based on a model of photon diffusion from a glowing source located at one of a plurality of depths, and having one of a plurality of sizes and shapes, through a turbid medium having absorption and scattering properties similar to those of biological tissue, and the unit is operable to (i) compare the quality of fit between each of
20 said plurality of theoretical light-intensity profiles and first measured light intensity profile, (ii) select the theoretical light intensity profile which provides the best fit to the first measured light intensity profile, and (iii) obtain an approximate depth and shape of the source in the subject using parameters from the theoretical light intensity profile selected in (ii).

25

30. The apparatus of claim 23, wherein the computational unit is operable to generate a visual 2-, or 3-dimensional representation of said light-emitting source, using the approximate depth and shape of the source in the subject, and superimposing said visual representation onto a 2- or 3-dimensional image of the
30 subject.

31. The method of claim 1, wherein said obtaining includes obtaining absolute intensity values.

5 32. A method of determining the depth of a light-emitting source in tissue of a subject, comprising:

(a) measuring light emission intensity from the subject at two or more different wavelengths between about 400 and about 1000 nm;

(b) obtaining information related to optical properties of subject's tissue, and

10 (c) using the measured light intensities and the obtained information to determine the depth of the light-emitting source.

33. The method of claim 32, wherein step (a) is carried out by measuring light intensities at each at two different wavelengths, step (b) includes measuring
15 the coefficients of attenuation and diffusion in tissue or material corresponding to that of the subject, and step (c) includes using said coefficients in a formula to estimate depth of said source.

34. The method of claim 33, wherein step (b) is carried out by
20 determining, at two or more different wavelengths, light intensities from by a light-emitting source located at each of a plurality of depths in tissue or material corresponding to that of the subject, and step (c) is carried out by matching the measured light intensities with reference light intensities determined at each of the plurality of depths.

25

35. The method of claim 34, wherein step (a) includes measuring a spectral profile of light intensities from the light-emitting source, step (b) is carried out by determining spectral profiles of light intensities from a light-emitting source at each of a plurality of depths, and step (c) is carried out by
30 matching the measured profile with the determined profiles, to identify a best curve fit.

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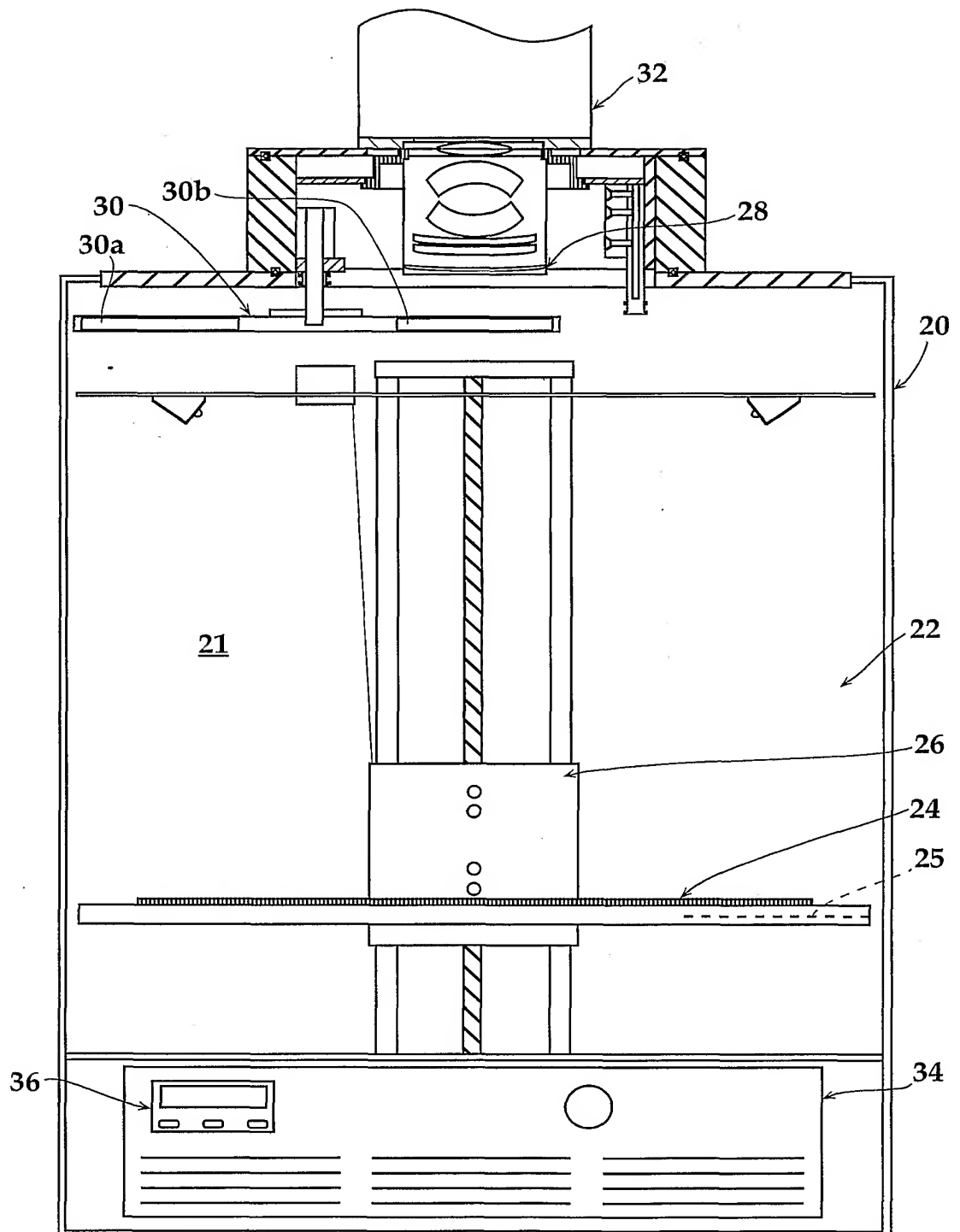
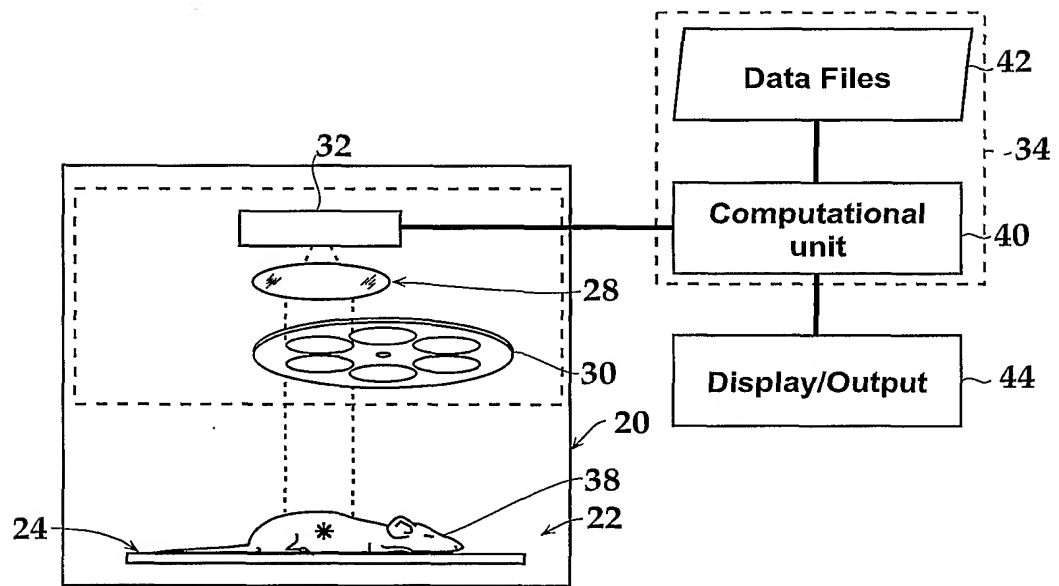
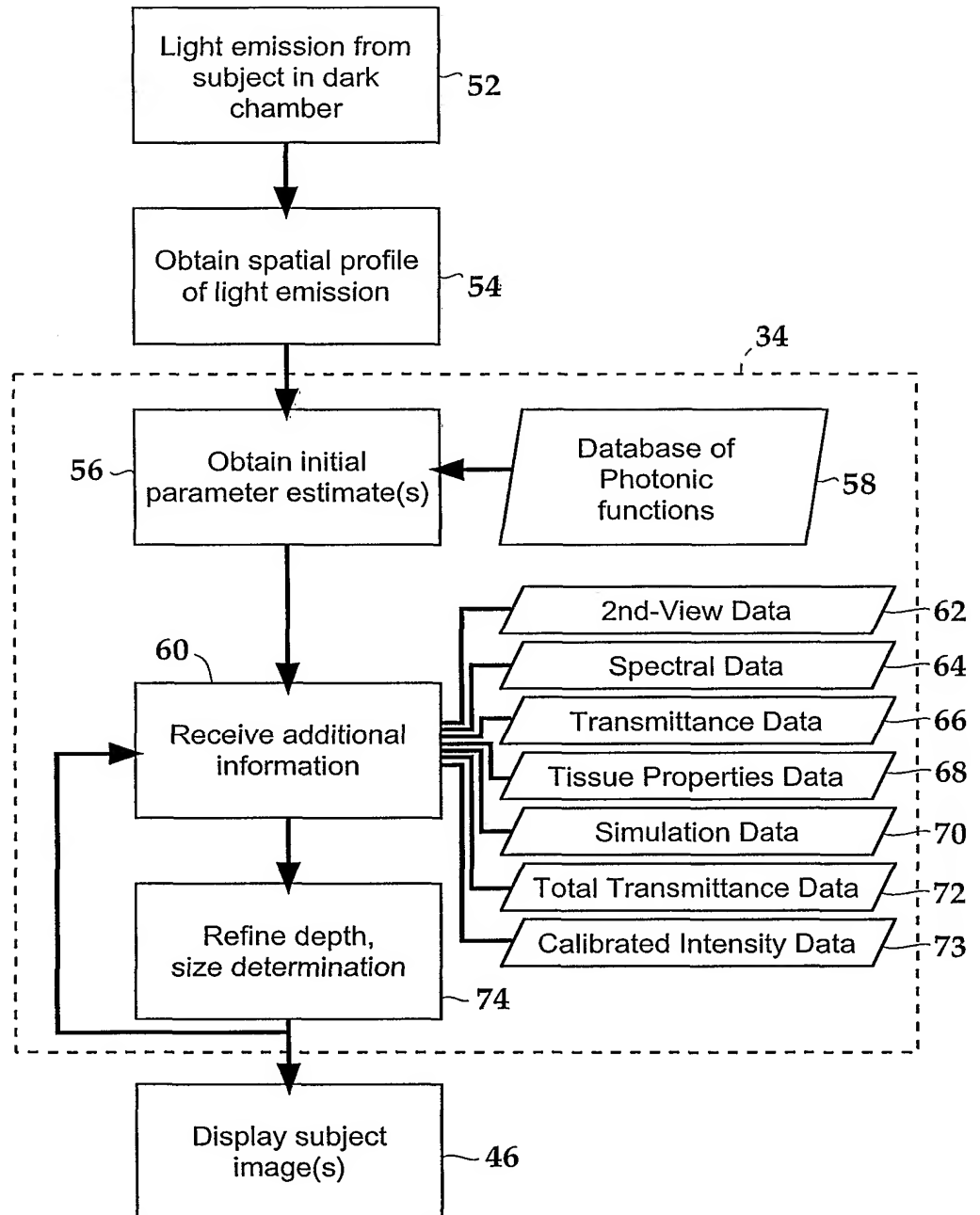


Fig. 1A

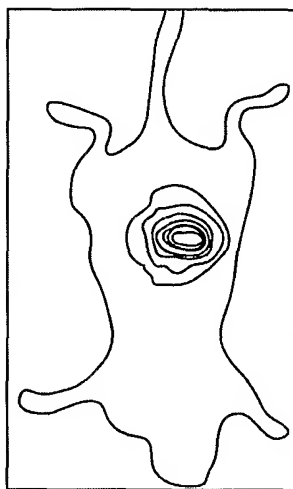
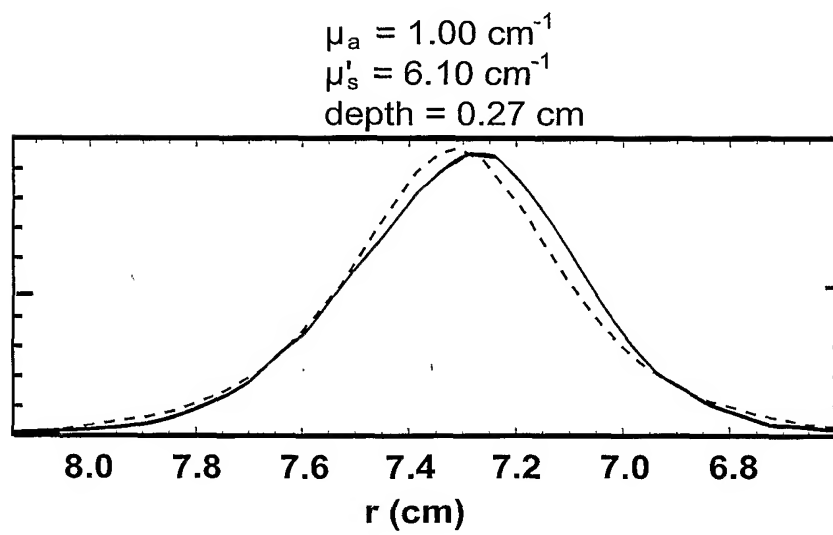
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**Fig. 1B**

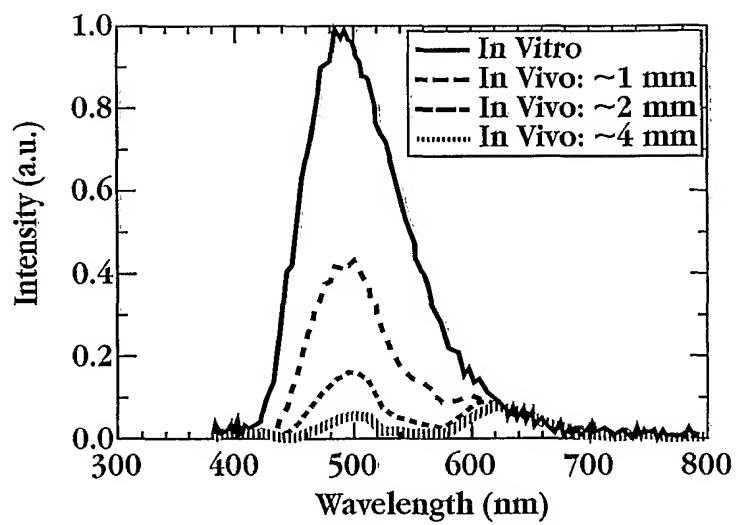
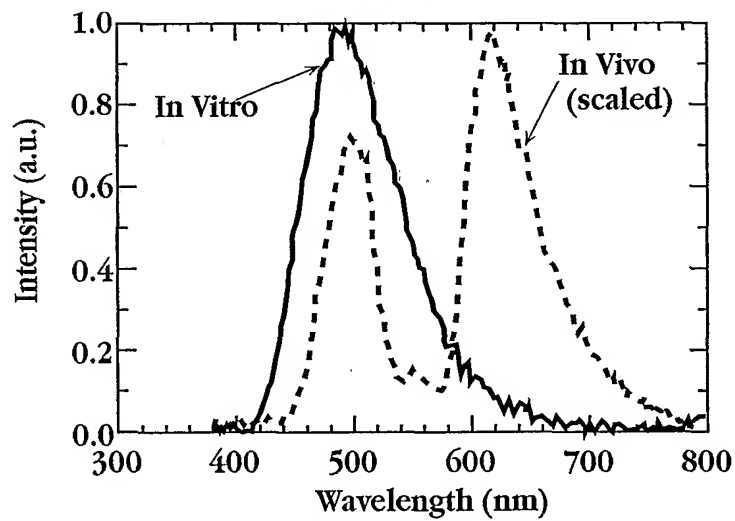
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**Fig. 2**

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**Fig. 3A****Fig. 3B**

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**Fig. 4A****Fig. 4B**

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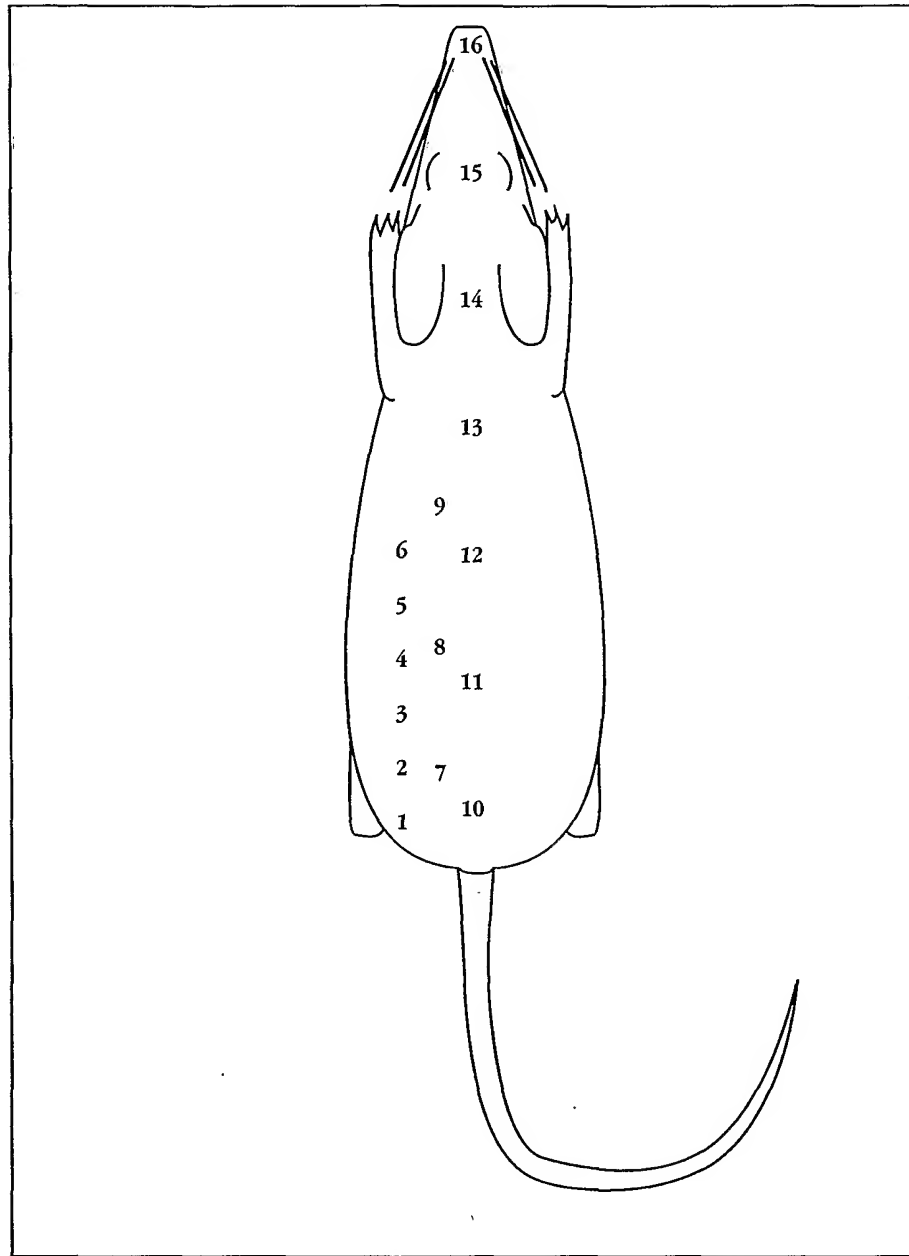
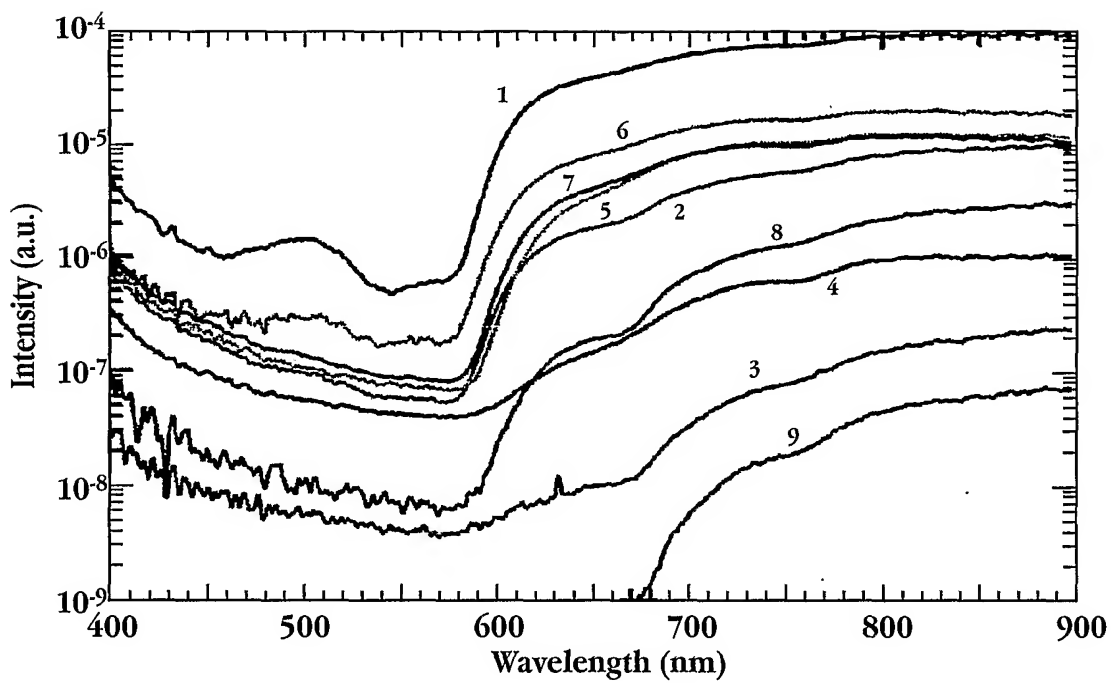
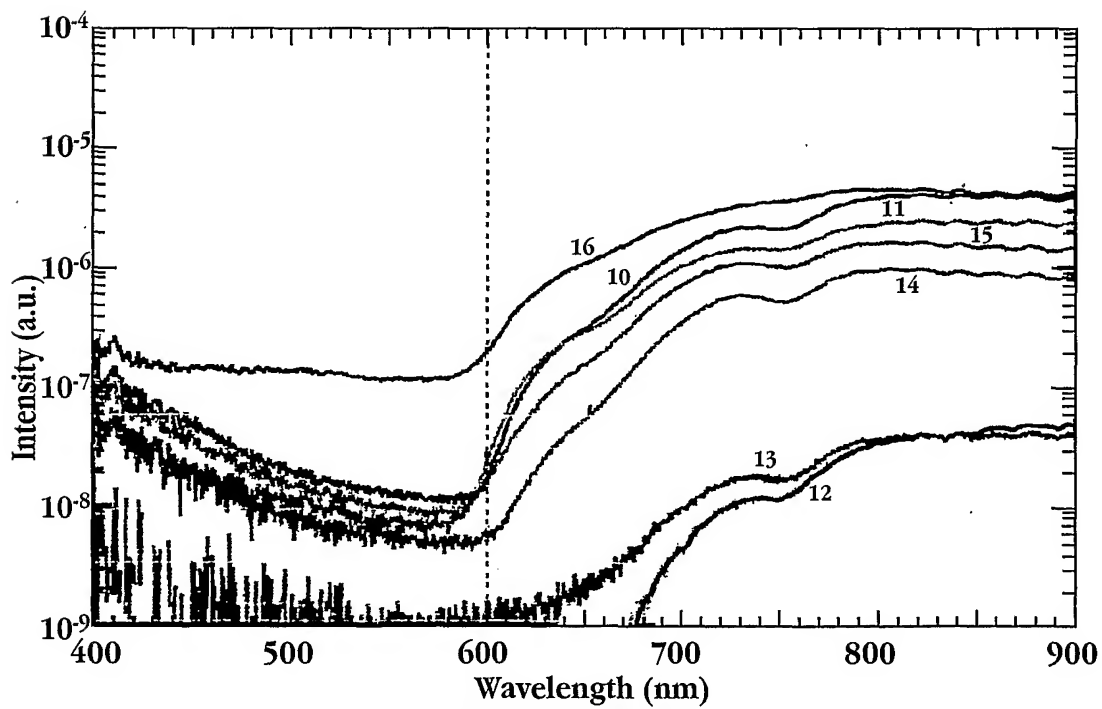
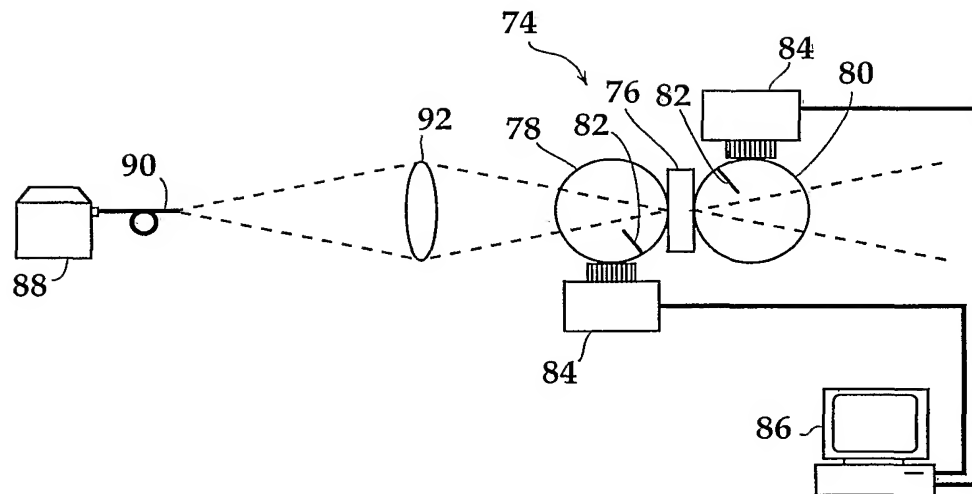
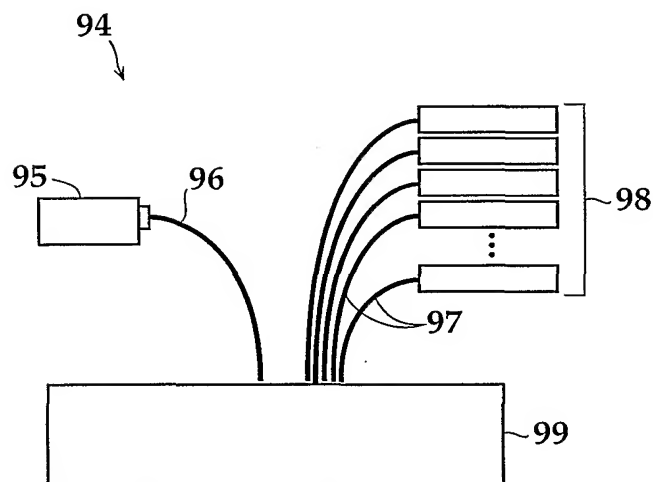


Fig. 5

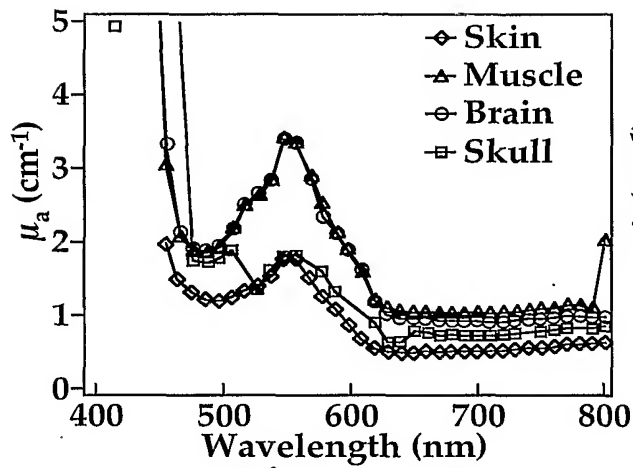
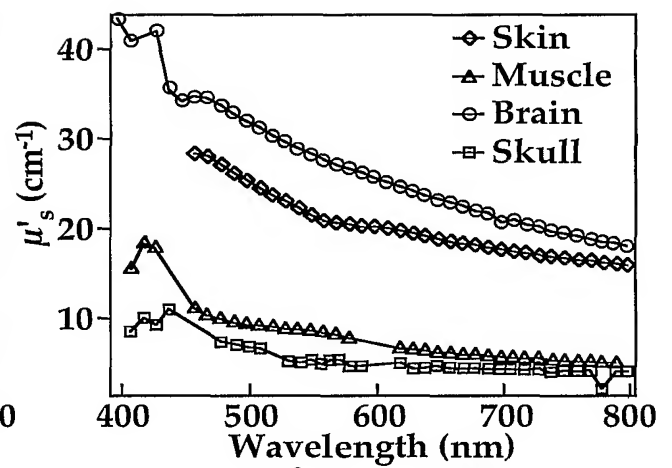
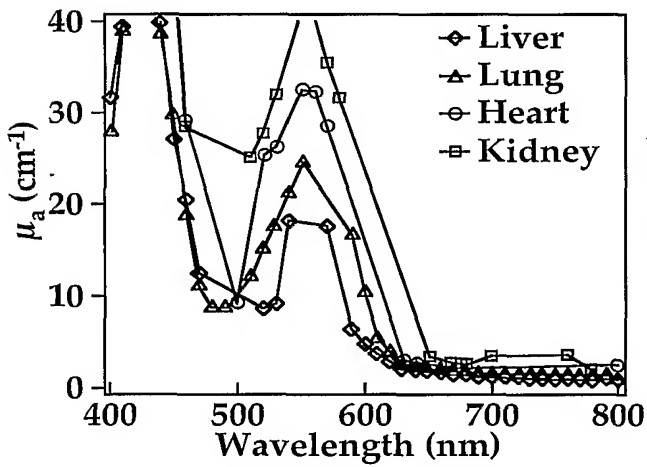
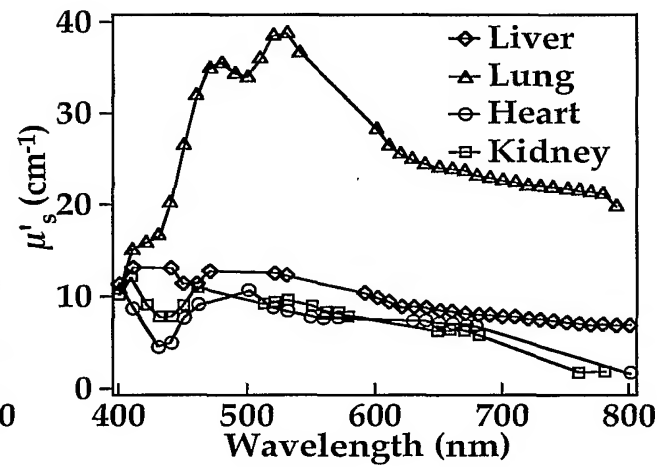
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**Fig. 6A****Fig. 6B**

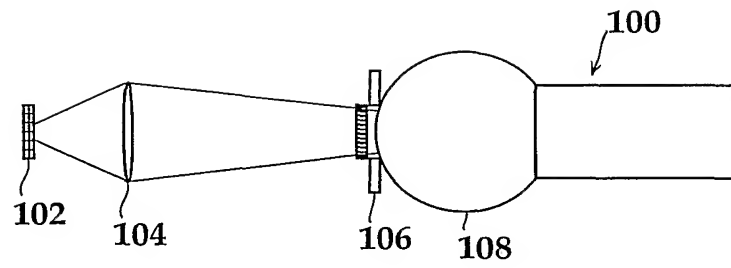
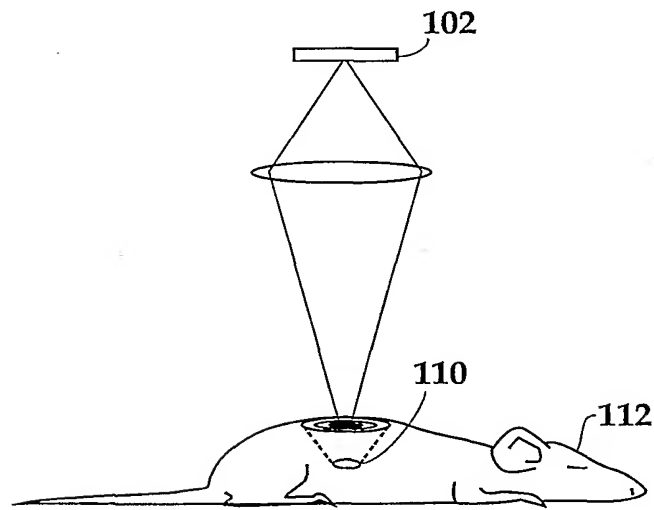
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**Fig. 6C****Fig. 6D**

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**Fig. 7A****Fig. 7C****Fig. 7B****Fig. 7D**

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**Fig. 8A****Fig. 8B**

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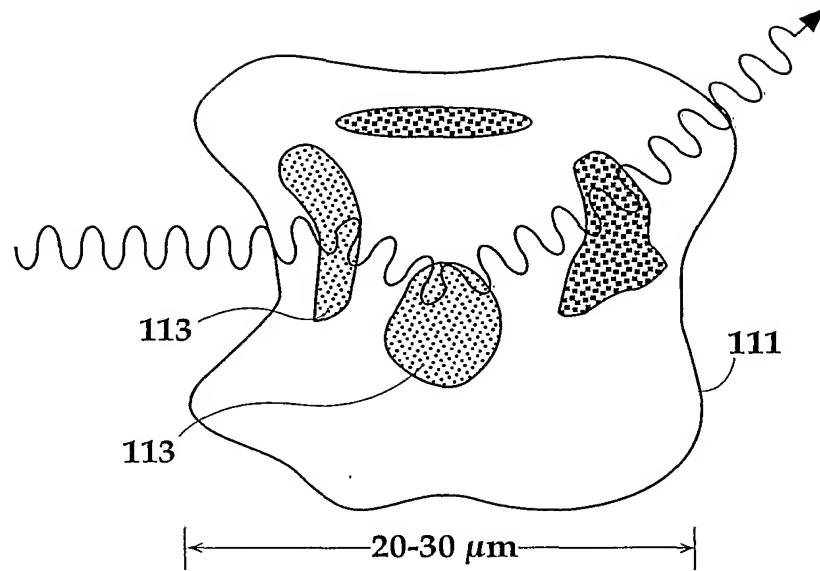


Fig. 9A

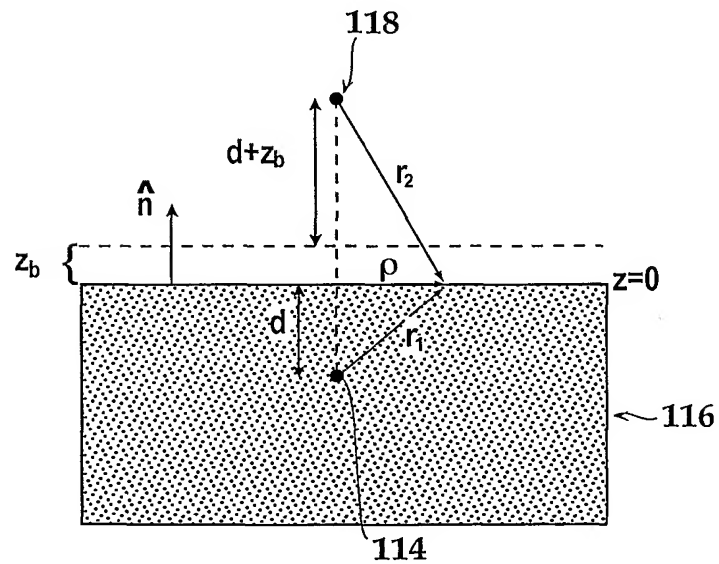


Fig. 9B

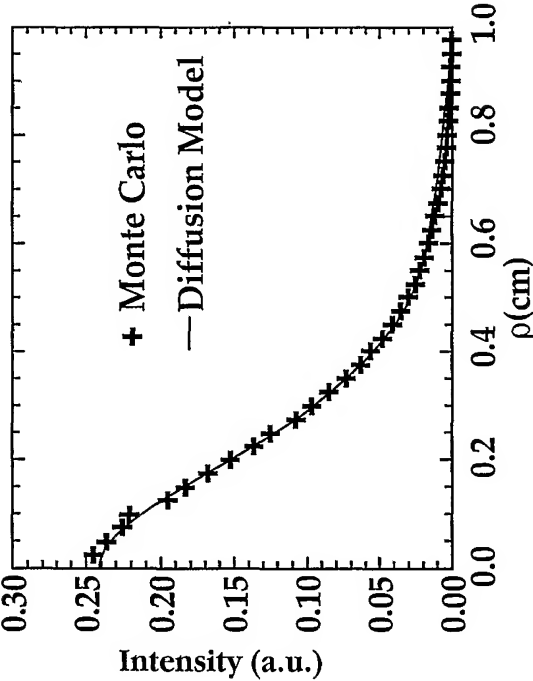


Fig. 10B

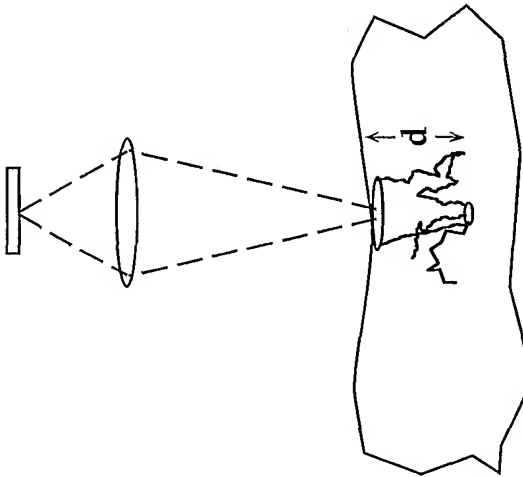


Fig. 10A

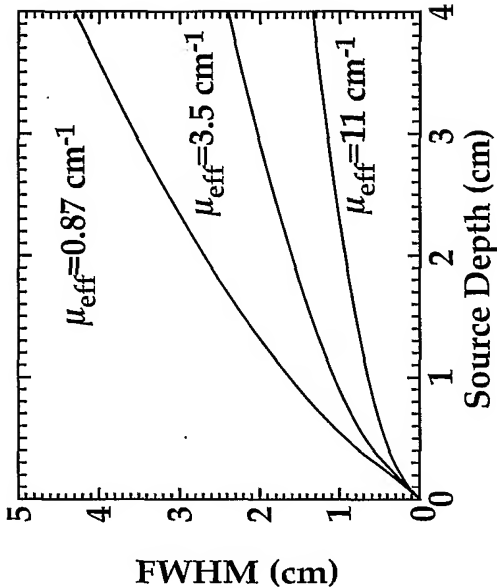


Fig. 11B

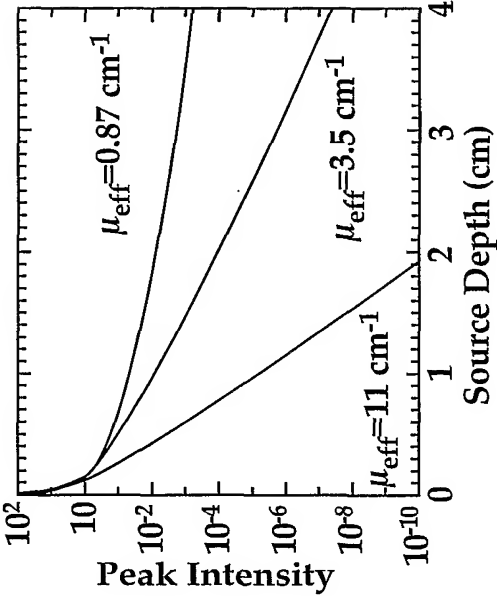
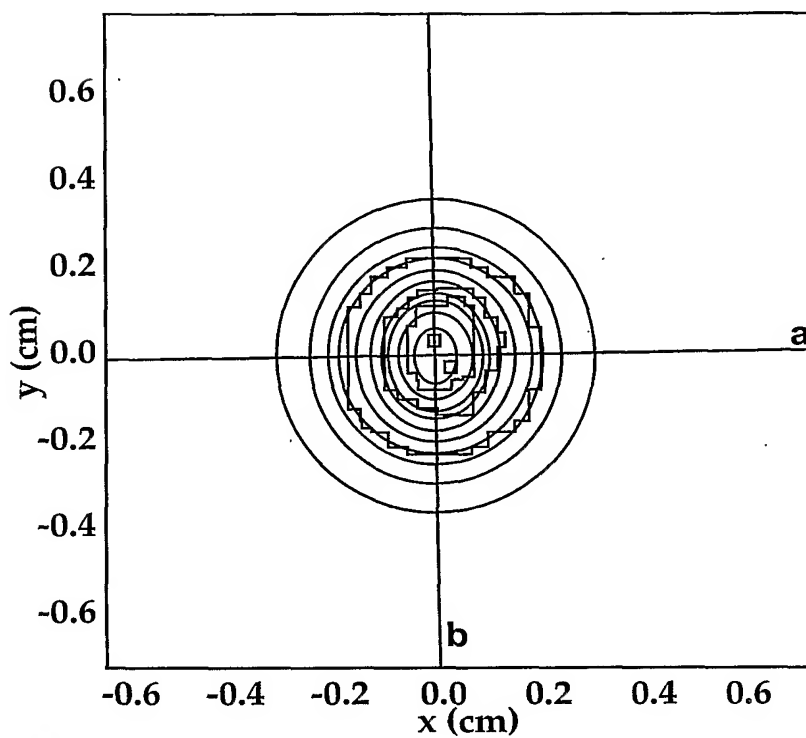
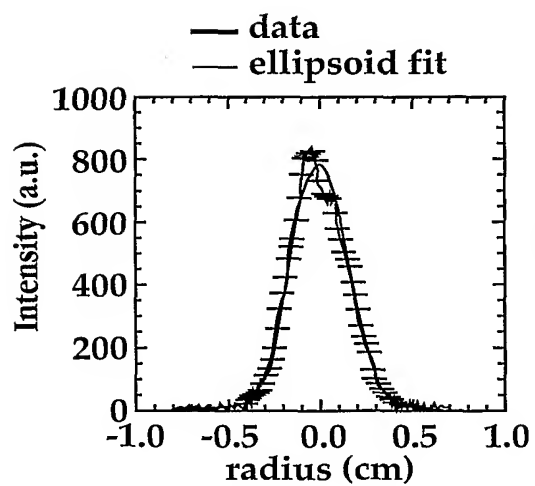
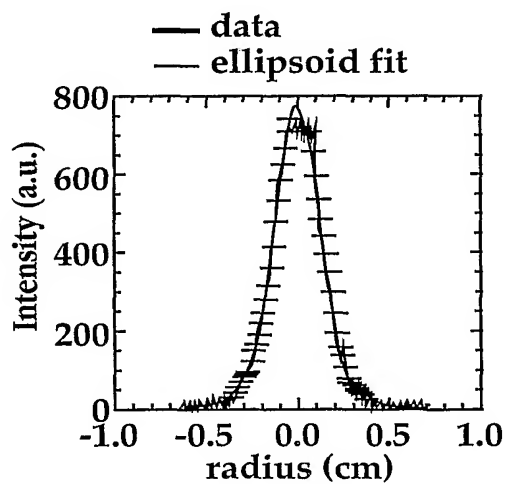
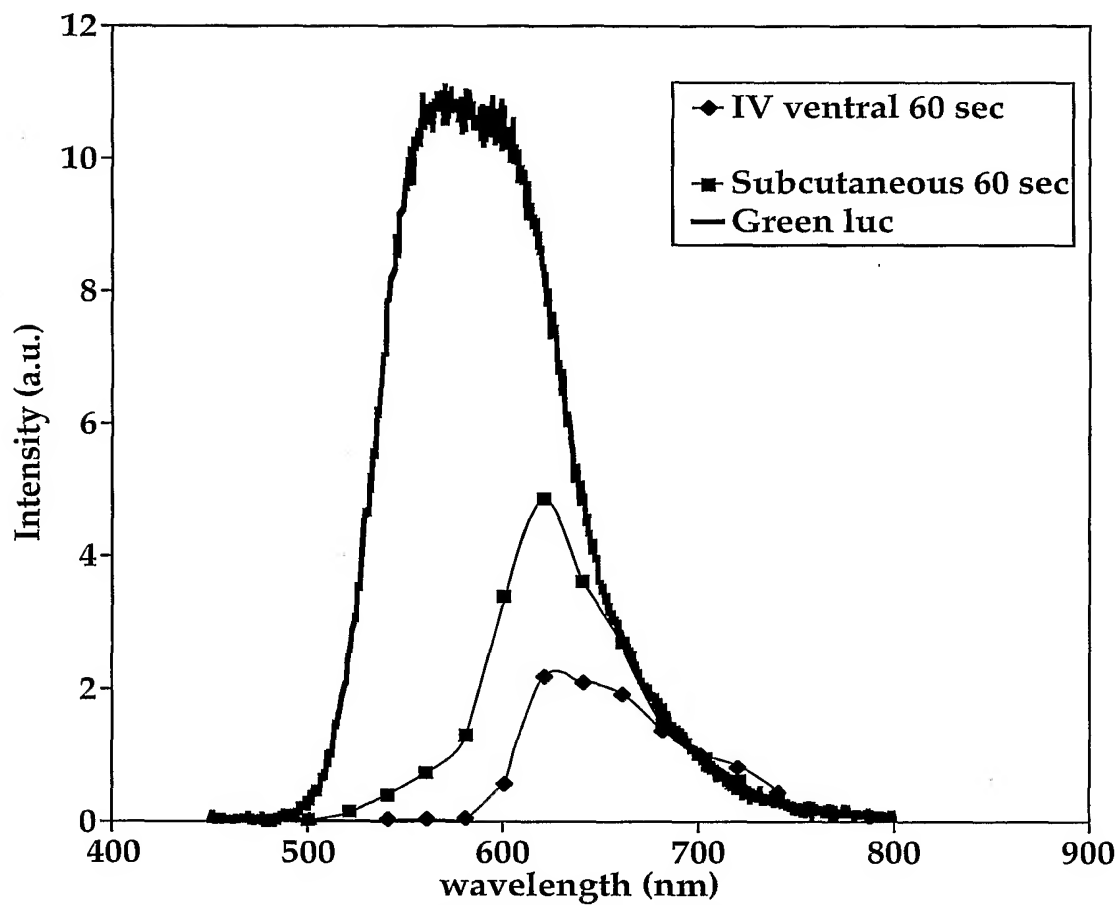
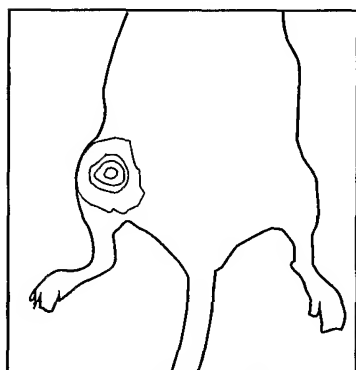
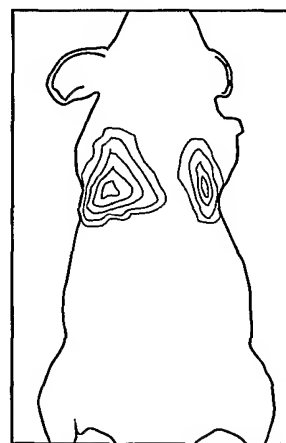


Fig. 11A

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**Fig. 12A****Fig. 12B****Fig. 12C**

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**Fig. 13A****Fig. 13B****Fig. 13C**